

Acta Microbiologica Hungarica

VOLUME 38, NUMBER 1, 1991

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Akadémiai Kiadó, Budapest

ACTA MICROBIOL. HUNG. AMHUEF 5 38 (1) 3-79 (1991) HU ISSN 0231-4622

ACTA MICROBIOLOGICA HUNGARICA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

Acta Microbiologica publishes reviews and original papers on microbiological subjects in English.

Acta Microbiologica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
H-1117 Budapest, Prielle K. u. 19—35.

Manuscripts and editorial correspondence should be addressed to

Acta Microbiologica
Institute of Microbiology, Semmelweis University Medical School
H-1445 Budapest, P.O. Box 370

Subscription information

Orders should be addressed to

KULTURA Foreign Trading Company
H-1389 Budapest P.O. Box 149

or to its representatives abroad

Acta Microbiologica Hungarica is abstracted/indexed in Abstracts of World Medicine, Biological Abstracts, Chemical Abstracts, Chemie-Information, Current Contents-Life Sciences, Excerpta Medica database (EMBASE), Index Medicus

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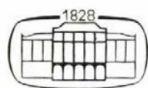
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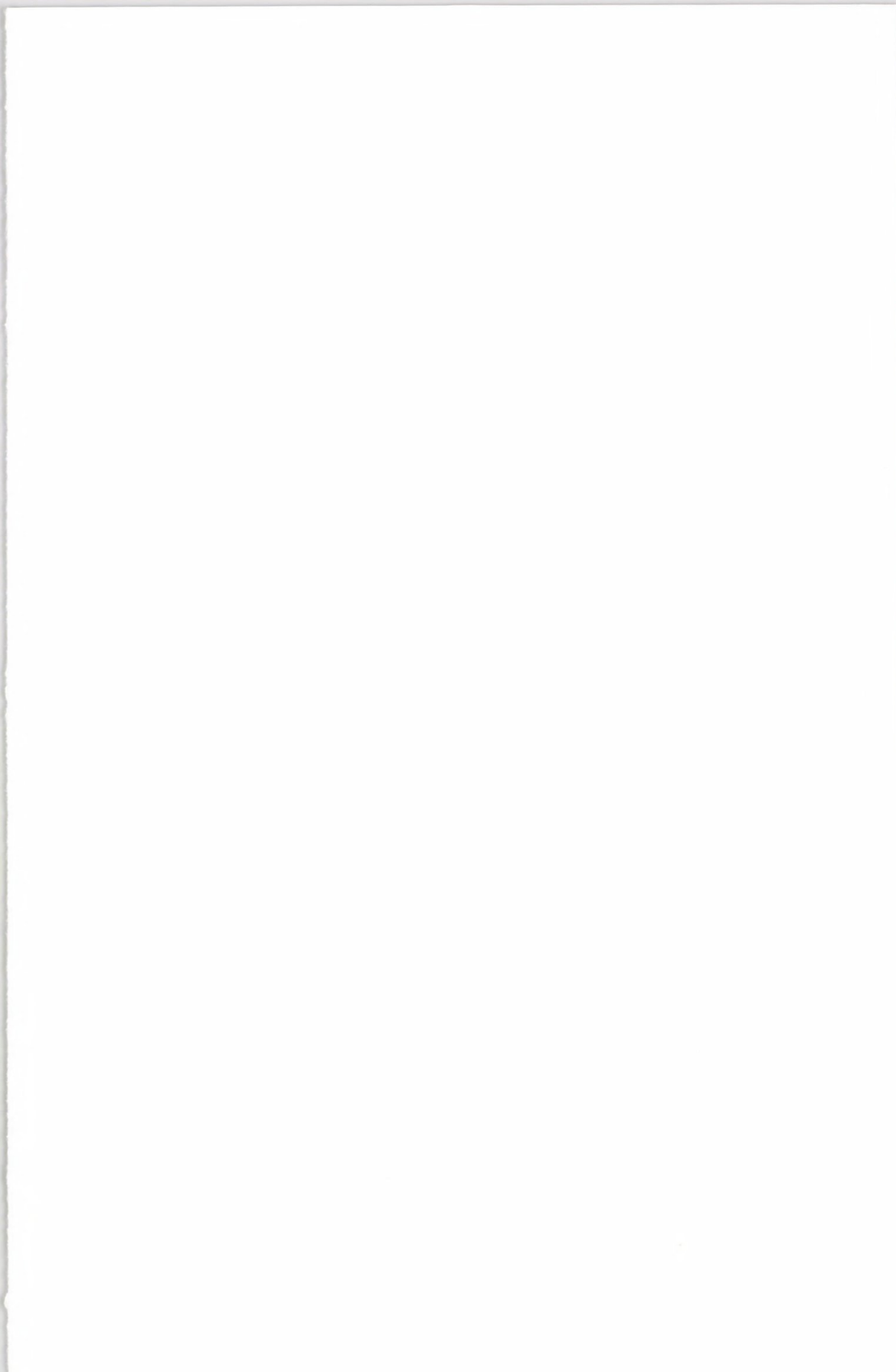
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HEAT RESISTANCE OF *LISTERIA MONOCYTOGENES* IN NATURALLY INFECTED AND INOCULATED COW'S MILK*

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(Received September 16, 1989)

A two phase slug flow tubular heat exchanger was used for the thermal inactivation of *Listeria monocytogenes* in natural infected milk from seven cows. *L. monocytogenes* serotype 4b inoculated UHT sterilized milk was monitored in a parallel study. The two milks were heated at 71.1 °C for holding times of 2, 4, 10, 15, 20 and 30 s. Milk was assayed for survivors immediately after heat treatment and weekly thereafter for 4 weeks during storage at 4 °C. No survivors were detected in the naturally infected milk at any of the holding times. Survivors were found at 2 and 4 s in the inoculated UHT milk with initial titres of 8×10^2 to 7.1×10^3 c.f.u./ml, only after storage at 4 °C for 28 days. No survivors were detected for 10 through 30 s holding times.

In the last years, according to the published data, the incidence of human foodborn listeriosis is becoming greater. Among other foods it has been found that pasteurized milk may also be a source of *Listeria monocytogenes* infections [1]. Therefore, the examination of thermal resistance of *L. monocytogenes* in milk was, from different pointes of view, a subject of a line of investigations [2–7].

Examinations described in this paper refer to thermal resistance of *L. monocytogenes* in naturally and artificially infected milk and on the revitalization of listeria after heat treatment.

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* Presented at the 10th International Symposium on Listeriosis, Pécs, Hungary, August 22–26, 1988.

Investigations were supported by the Yugoslavia-US Joint Board for scientific and technological cooperation, Project YO-ARS-90-JB, Pp 636.

Materials and methods

Naturally infected milk from 7 cows shedding *L. monocytogenes*, was used for examination of thermal resistance.

Artificial contamination was performed on UHT sterilized milk with 3.2% of fat content which was inoculated with fresh *L. monocytogenes* 4b strain at final concentrations of 10^2 – 10^3 . Milk samples were exposed to 71.7 °C using a two phase slug flow tubular heat exchanger for 2, 4, 10, 15, 20 and 30 s. Immediately after the heat treatment bacteriological examination of the samples was performed, and afterwards every 7 days during 4 weeks, at the storage on 4 °C.

Results

Repeated bacteriological examinations of raw milk from 40 cows, at which we obtained positive findings for *L. monocytogenes* by previous examinations (bacteriological from uterine mucose, serological or allergenic test), listeriae were occasionally isolated directly from milk of 2 cows at low concentrations/ml (4.8×10^2 and 3.1×10^2), and through the enrichment broth listeriae were isolated from milk of 5 cows.

Results given in Table I, referring to naturally infected milk with *L. monocytogenes* after heat treatment at 71.7 °C for 2, 4, 10, 15, 20 and 30 s, show that there was no survival of listeriae in any of the cases. *Listeriae* were not isolated either on the 7th, 14th or 21st day of storage after heat treatment.

Artificially contaminated milk with initial concentrations of listeriae/ml of 10^2 – 10^3 (Table II) immediately after heat treatment, the survival of *L. monocytogenes* was confirmed only in 2 cases at 2 s treatment. However, during the storage at 4 °C up to 28 days, there appeared a growth of listeriae in samples treated for 2 and 4 s. This phenomenon did not appear at treatment for 10, 20 and 30 s. Survived listeriae showed growth during the storage so that after 28 days the number of listeriae went up to log 7.

Table I

L. monocytogenes in naturally infected cow's milk and its survival at 71.7 °C
(number of listeria in ml)

Cow No.	Direct plating	After incubation in enrichment broth	After heat treatment for 20–30	After storage at 4 °C		
				7	14	21
1	4.8×10^2	+	0	0	0	0
2	—	+	0	0	0	0
3	—	+	0	0	0	0
4	—	+	0	0	0	0
5	—	+	0	0	0	0
6	—	+	0	0	0	0
7	3.1×10^2	+	0	0	0	0

— Not detectable in direct plating

+

 Detectable after incubation in enrichment broth

Table II

Heat inactivation of *L. monocytogenes* at 71.7 °C in artificially contaminated UHT milk
(number of listeria in ml)

Heating time, (s)	No. of run	<i>L. monocytogenes</i> after storage at 4 °C (days)				
		0	7	14	21	28
0	1	7.1×10^3	1.4×10^5	1.5×10^9	7.2×10^7	3.5×10^{10}
	2	5.2×10^3	2.5×10^4	7.3×10^6	2.8×10^7	3.0×10^{10}
	3	8.0×10^2	4.2×10^4	1.5×10^6	1.0×10^7	4.6×10^9
	4	2.0×10^3	8.3×10^4	5.3×10^6	2.4×10^7	2.3×10^9
	5	3.0×10^3	3.6×10^5	6.2×10^6	7.3×10^6	9.8×10^8
2	1	1	2.6×10^2	2.3×10^4	5.0×10^5	1.9×10^7
	2	0	6	8.0×10^1	1.6×10^2	1.3×10^4
	3	0	1	2.5×10^2	4.8×10^2	6.0×10^4
	4	1	20	6.5×10^2	1.2×10^3	2.1×10^4
	5	0	21	7.0×10^2	3.0×10^3	4.0×10^4
4	1	0	3	10	4.3×10^3	5.4×10^6
	2	0	2	7.0×10^1	8.5×10^2	6.0×10^3
	3	0	0	0	0	2
	4	0	8	2.3×10^2	3.2×10^3	4.6×10^4
	5	0	2.6×10^2	5.3×10^2	7.5×10^3	6.2×10^4
10	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0	0	0	0	0
15	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0	0	0	0	0
20	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0	0	0	0	0
30	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	0	0	0	0	0

Discussion and conclusions

Naturally infected milk of cows occasionally shedding listeriae in their milk at low concentrations was examined. Milk was bacteriologically examined and it had also undergone heat treatment few hours after milking. Since the half life of bovine neutrophils, i. e. phagocytic cells is about 6–8 h, in our examinations listeriae were in an intracellular state as well as free at the same time.

The results show that the heat treatment at 71.7 °C for 2 and 4 s of artificially contaminated milk causes a heat shock at a certain number of listeriae which did not have the ability to grow immediately after treatment. Only after storage at 4 °C these microorganisms recover and an obvious growth can be obtained.

In our previous investigations [7] survival of *L. monocytogenes* in milk with high initial concentrations, higher than 10^7 , was confirmed at heat treatment for 15 s. It appears that during the commercial pasteurization of milk infected by high initial number some *L. monocytogenes* cells could survive and their revitalization during the storage of pasteurized milk could be expected.

The following conclusions may be drawn.

(i) *L. monocytogenes* in naturally infected milk with maximum 10^3 cells did not survive heat treatment at 71.7 °C for 2, 4, 10, 15, 20 and 30 s.

(ii) In artificially infected milk with initial concentrations of *L. monocytogenes*/ml up to 10^3 , the presence of listeriae was confirmed only at treatment for 2 s in two cases. No survivors were detected for 10 through 30 s holding times.

(iii) Heat treatment at 71.7 °C for 2 and 4 s causes heat shock at a certain number of listeriae. These microorganisms have the ability to multiply during the storage at 4 °C, which was confirmed by examination after 7, 14 and 28 days.

REFERENCES

1. Fleming, D. W., Cochi, S. L., MacDonald, K. L. et al.: N Engl J Med **312**, 404 (1985).
2. Bradshaw, J. G., Peeler, J. T., Corwin, J. J., Hunt, J. M., Tierney, J. T., Larkin, E. P., Twedt, R. M.: J Food Prot **48**, 743 (1985).
3. Bunning, V. K., Crawford, R. G., Bradshaw, J. G., Peeler, J. T., Tierney, J. T., Twedt, R. M.: Appl Environ Microbiol **52**, 1398 (1986).
4. Bunning, V. K., Donnelly, C. W., Peeler, J. T., Briggs, E. H., Bradshaw, J. G., Crawford, R. G., Beliveau, C. M., Tierney, J. T.: Appl Environ Microbiol **54**, (1988).
5. Doyle, M. P., Glass, K. A., Beery, J. T., Garcia, G. A., Pollard, D. J., Schultz, R. D.: Appl Environ Microbiol **53**, 1433 (1987).
6. Fernandez, J. F., Dominguez, J. F., Boland, L., Ferri, J. A. V., Dieste, E. F. P., Canelo, V. B., Fernandez, J. L. B.: J Appl Microbiol **63**, 553 (1987).
7. Kovičević, I., Vujičević, I. F., Štajner, B., Galić, M., Vulić, M.: In Courtieu, A. L. (ed.): Proc. 9th International Symposium on the problems of listeriosis, Nantes, 1985. Université de Nantes, Nantes 1986, pp. 356-359.

DISTRIBUTION OF NITRIFYING BACTERIA IN FISH CULTURE TANKS: INFLUENCE OF MANAGEMENT FACTORS

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Examination of microbial populations of ammonia and nitrite oxidizers in model tanks used for farming of air-breathing catfish (ABFFS), polyculture of herbivorous carps (PS) and control system (CS) revealed maximum abundance of ammonia and nitrite oxidizers in ABFFS and minimal in CS. In any of the two culture systems, the chemical concentration or density of bacteria developed due to feed application was many times higher than other management factors. At a given time, the bacteria of each group occurring in any management protocol of ABFFS was about 10% higher than PS. Culture of air-breathing fishes caused greater abundance of ammonia and nitrite oxidizers than the herbivorous carps. There was a strong positive correlation between each of the chemical concentration of produced nitrite (ammonia oxidizers) and density of nitrite oxidizers and ratio of total nitrogen to phosphate.

One of the major problems in fish culture is the turnover and removal of large amounts of nitrogenous compounds resulting from massive input of nitrogen-rich feeds and pond fertilization. Major part of the feed nitrogen is excreted by the fish as ammonia [1, 2]. In addition, decomposition of unconsumed feed and the use of manure and nitrogen fertilizer further add ammonia to the fish ponds. Ammonia can be highly toxic to fish [3], mainly at high pH where it exists in its unionised form. Not only ammonia but also nitrite-N is toxic to many species of fishes [4].

In natural waters, ammonia and nitrite are eventually transformed into nitrate by the nitrifying bacteria. Most of the ammonia produced in the fish pond throughout the growth period was trapped and stored in the sediment, and was found to be particle bound [5]. It is thus important to study and characterize the cyclic changes of nitrifying bacterial populations in relation to the concentration of nitrogenous compounds throughout the year. Nitrifying bacteria are capable of oxidizing NH_4^+ to NO_2^- in the first step, and NO_2^- to NO_3^- in the next step. The first step is restricted to the genus *Nitrosomonas*, although some other genera (*Nitrosococcus*, *Nitrospira* and *Nitrosobolus*) are known to perform this process [6, 7]. *Nitrobacter* spp. are largely responsible for the oxidation of nitrite to nitrate. Engel (cited by [8]) demonstrated the

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presence of the chemoautotrophic ammonia oxidizers (*Nitrosomonas europaea*) as well as nitrite oxidizers (*Nitrobacter winogradskyi*) in large numbers and stressed the quantitative significance of autotrophic nitrification in inland waters. Although the presence of heterotrophic nitrification has been demonstrated only in laboratory culture, its importance in aquatic system is, as yet, unresolved [9-11].

The management protocol of fish farming has a profound influence on the nitrogen dynamics of a pond environment. The culture of carnivorous catfish and herbivorous carps is likely to cause a marked difference in the nitrogen input and hence the nitrifying bacterial load. The purpose of this study was to examine the changes of nitrifying bacterial load and nitrogen concentration as affected by the farming of carnivorous and herbivorous fish in model tanks under tropical conditions.

Material and methods

Model tanks. The chemical concentration of nitrite produced by ammonia oxidizers and the density of nitrite oxidizers were determined in waters of model tanks ($2.85 \times 1.17 \times 1.96$ m) used for farming of carnivorous air-breathing catfishes (*Clarias batrachus* and *Heteropneustes fossilis*) (ABFFS) and polyculture of herbivorous carps (*Labeo rohita*, *Catla catla*, *Cirrhinus mrigala* and *Cyprinus carpio*) (PS). Tank without fish and feed was used for control systems (CS). Each treatment was always quadruplicated. The results of the study were confirmed by two annual experiments conducted during the period of March through February in two consecutive years (1982 to 1984). The management protocol followed in this study were described earlier [12].

To quantify the bacterial load developed due to various management factors such as isolated effects of fish metabolites and feed as well as their combined effect, an outdoor experiment was conducted in sixteen tanks (300 l capacity) under four conditions in quadruplicate using the fish stocking load of 6000 kg ha⁻¹. Test fishes were introduced into the first group, the second batch received only feed in the form of fish meal (10% of the fish load), whereas the third group was provided with both fish load and feed; the fourth contained neither fish load nor feed (control). Water samples were examined by collecting the samples at biweekly intervals during the culture period of 15 days.

The concentration of produced nitrite (ammonia oxidizers) was determined in Meiklejohn's broth medium [2] incubated at $30^\circ\text{C} \pm 1^\circ\text{C}$ initially for 15 days. A relatively high incubation temperature was used considering the tropical conditions of the experimental tanks. The nitrifiers that grew in this medium served as inoculum for the next incubation period. After 4 weeks of incubation, the chemical concentration of produced nitrite (ammonia oxidizers) in the broth medium was determined spectrophotometrically at 540 nm on treatment with Griess-Ilosvay reagent [13] and expressed in $\mu\text{g ml}^{-1}$ [14]. Lack of pure culture of ammonia oxidizers did not permit to use a calibration curve for expressing the results in counts ml⁻¹, and comparing with counts of nitrite oxidizers (counts ml⁻¹).

The Meiklejohn's broth medium contained (g/l): NaCl, 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.14; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; redistilled water, 90 ml; $(\text{NH}_4)_2\text{SO}_4$, 0.66; KH_2PO_4 (0.1 molar solution), 10 ml. The medium was diluted by adding distilled water up to 1 l, and then 10 g of powdered CaCO_3 was added and 0.4 ml of microelement solution containing ($\mu\text{g/l}$) M, 22; B, 21; Cu, 17; Zn, 16; Co, 14.

For enumeration of nitrite oxidizers the MPN technique [7] was used at $30^\circ\text{C} \pm 1^\circ\text{C}$ in the dark for 28 days. Stock solutions were prepared in distilled water (g/100 ml) using the following chemical constituents: KNO_3 , 0.85; $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 1.34; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.0; K_2HPO_4 (0.2 M), 3.48; KH_2PO_4 (0.2 M), 2.72; chelated iron ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.246; EDTA di-sodium, 0.331); trace elements ($\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.01; MnCl_2 , 0.02; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.0002; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.002). From these stock solutions, the following were taken to prepare the medium (-/l): KNO_3 , 1.0; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0;

K_2HPO_4 (0.2 M), 4.0; KH_2PO_4 (0.2 M), 1.0; chelated iron, 1.0; trace elements, 1.0; pH 7.2 to 7.5. The calcium chloride and magnesium sulphate were combined, autoclaved (at 121 °C for 15 min) separately and added aseptically to the sterile solution of remaining ingredients. Spot test as described by [7] was performed to check for the disappearance of nitrite-N.

Statistical analysis. The physico-chemical variables of water were monitored at regular intervals from all the fish culture tanks following the appropriate procedures described in standard methods [15]. Initially a two way analysis of variance was applied to compare each of the chemical concentration of produced nitrite (ammonia oxidizers) and density of nitrite oxidizers among three culture systems employed. The results of the ANOVA yielded significant effect of interactions between the farming management and season for each group of bacteria. Therefore, one-way analysis of variance was used to compare either the chemical concentration of produced nitrite (ammonia oxidizers) or density of nitrate oxidizers among three culture systems employed. This was computed using the data of each group of bacteria in three systems on each date of observation. A standard method described in Snedecor and Cochran [16] has been used for analysis of variance. The relationships between the bacterial load and some of the selected water quality parameters were determined by using correlation coefficients with $N = 24$ at 0.05 level of significance for each treatment.

Results

The physico-chemical variables of water are summarized in Table I.

Distribution of bacteria in fish culture tanks. The chemical concentration of produced nitrite (ammonia oxidizers) was highly variable among three variants (Fig. 1). The mean concentration ranged from $0.051 \mu\text{g ml}^{-1}$ in CS to $0.118 \mu\text{g ml}^{-1}$ in ABFFS. In carp culture tanks (PS) the concentrations ($0.094 \mu\text{g ml}^{-1}$) ranged between the two extremes. One way analysis of variance of the data of concentration of produced nitrite (ammonia oxidizers) revealed significantly lower concentration in the control system than in the remaining two culture systems employed ($F_{2,6} \geq 17.21$; $P < 0.05$) during all data of measurement except on two occasions (Table II). The load of bacteria observed in the ABFFS was significantly higher than PS in most of the cases (Table II).

Despite the general seasonal trend, the magnitude of peak of bacteria differed markedly among three culture systems (Fig. 1). The peak was ill-defined in the CS, moderate in the PS, but was greatly enlarged in the ABFFS apparently due to the high nitrogen input in the system concerned.

The spatial distribution of the density of nitrite oxidizers (Fig. 1) followed the trend similar to that of chemical concentration of produced nitrite (ammonia oxidizers). The counts for nitrite oxidizers were maximum (15.6×10^2 cells ml^{-1}) and minimum (1.14×10^2 cells ml^{-1}) in the ABFFS and CS, respectively. One way variance analysis of density of nitrite oxidizers also revealed significant differences ($F_{2,6} \geq 25.08$; $P < 0.05$) from one system to another. The monthly counts of nitrite oxidizers in ABFFS were significantly higher than the remaining two systems in most of the cases.

The seasonal trends of the concentration of produced nitrite (ammonia oxidizers) as well as density of nitrite oxidizers were almost similar in PS and CS. A peak was exhibited during January and depression in May, but no such depression was encountered in ABFFS. The distribution patterns of chemical

Table I
Summary of the physico-chemical factors of water

Parameters	ABFFS		PS		CS	
	Range	Overall mean	Range	Overall mean	Range	Overall mean
Temperature ($^{\circ}\text{C}$)	21.0–35.0	28.3	21.0–35.0	28.3	21.0–35.0	28.3
pH	7.26–7.81	7.55	7.34–7.89	7.62	7.45–8.17	7.71
Free CO_2 (mg l^{-1})	12.0–41.5	24.7	12.0–36.0	20.25	5.0–31.0	17.5
HCO_3^- (mg l^{-1})	156.0–437.7	312.4	164.0–427.0	287.2	138.0–415.0	261.5
Dissolved Oxygen (mg l^{-1})	4.0–14.0	11.06	7.2–17.6	12.5	8.2–18.0	14.4
$\text{NH}_4\text{—N}$ (mg l^{-1})	0.44–5.22	1.55	0.26–4.52	1.35	0.21–4.13	0.96
$\text{NO}_2\text{—N}$ (mg l^{-1})	0.05–0.131	0.077	0.004–0.17	0.065	0.002–0.11	0.029
$\text{NO}_3\text{—N}$ (mg l^{-1})	0.1–1.95	0.79	0.08–1.95	0.66	0.04–1.43	0.46
Kjeldahl—N (mg l^{-1})	2.8–18.7	11.3	1.75–17.9	10.5	1.05–14.3	9.25
$\text{PO}_4\text{—P}$ (mg l^{-1})	0.04–0.294	0.134	0.03–0.268	0.124	0.03–0.256	0.098
Specific conductivity ($\mu\text{mhos cm}^{-1}$)	0.355–0.666	0.546	0.369–0.658	0.543	0.309–0.608	0.486

Each figure represents the range and overall mean ($N = 48$) of two annual cycles (1982–84)

Table II
*Comparison of monthly mean chemical concentrations of produced nitrite (ammonia oxidizers) amongst the three systems by the Critical Difference (CD) test**

Months	Variance ratio (df = 2.6)	C.D. values at 5%	Mean concentrations			Variance ratio (df = 2.6)	C.D. values at 5%	Mean concentrations		
			ABFFS	PS	CS			ABFFS	PS	CS
March	33.89	0.017	0.08	0.065	0.03	147.15	0.017	0.125	0.12	0.03
April	183.19	0.007	0.06	0.036	0.015	39.04	0.011	0.055	0.065	0.03
May	106.78	0.006	0.0405	0.025	0.0095	108.31	0.008	0.045	0.041	0.0085
June	43.33	0.033	0.135	0.11	0.03	23.84	0.029	0.11	0.065	0.04
July	273.27	0.013	0.12	0.11	0.02	72.99	0.018	0.09	0.066	0.015
August	16103.50	0.001	0.089	0.08	0.02	131.20	0.007	0.051	0.035	0.009
September	39.039	0.017	0.125	0.095	0.07	5.46	0.052	0.091	0.085	0.035
October	23.26	0.026	0.11	0.10	0.05	17.21	0.029	0.125	0.09	0.065
November	378.65	0.006	0.129	0.105	0.07	53.58	0.014	0.116	0.085	0.065
December	12.96	0.065	0.25	0.19	0.13	85.27	0.016	0.145	0.099	0.070
January	58.33	0.039	0.305	0.205	0.155	22.62	0.052	0.220	0.145	0.095
February	32.13	0.017	0.117	0.14	0.09	27.03	0.011	0.095	0.08	0.065
Mean			0.130	0.106	0.057			0.106	0.081	0.044
Overall mean		0.118				0.094		0.051		

The chemical concentration was expressed in $\mu\text{g ml}^{-1}$.

* Horizontal lines indicate no significant difference ($P > 0.05$)

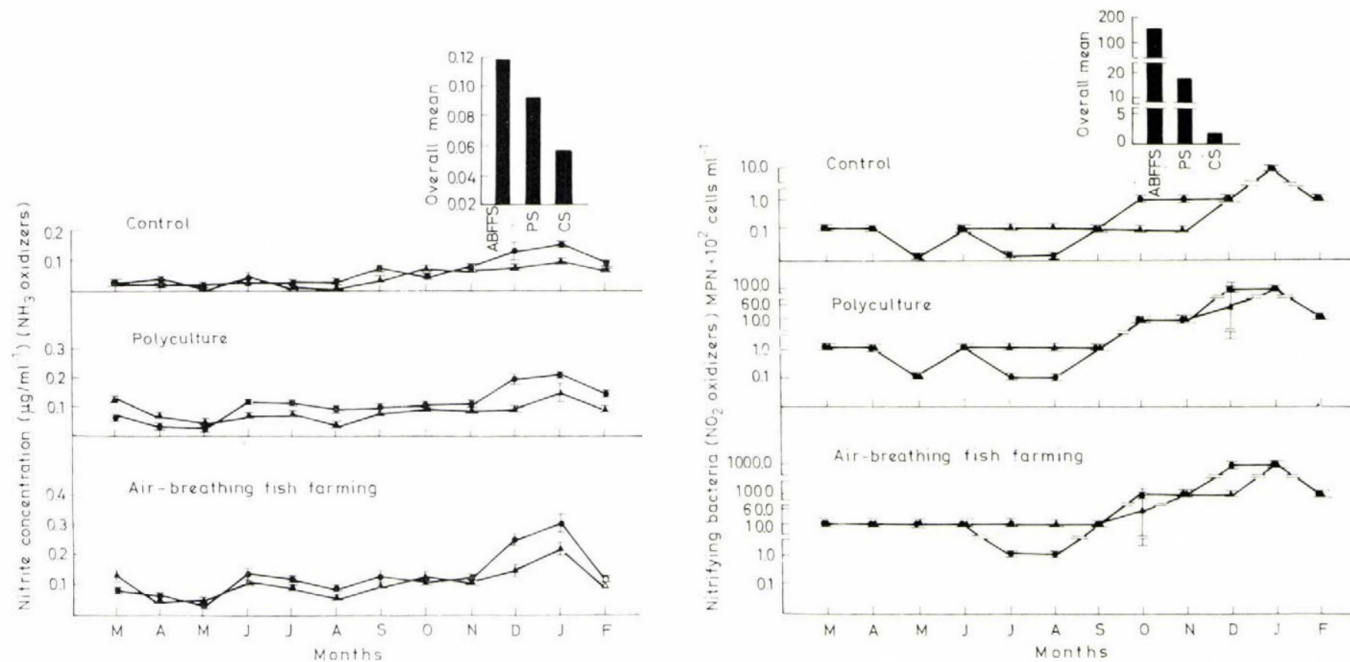


Fig. 1. Temporal course of chemical concentration of produced nitrite (ammonia oxidizers) and density of nitrite oxidizers in three culture systems in the long term study. ● 1982-1983; ▲ 1983-1984

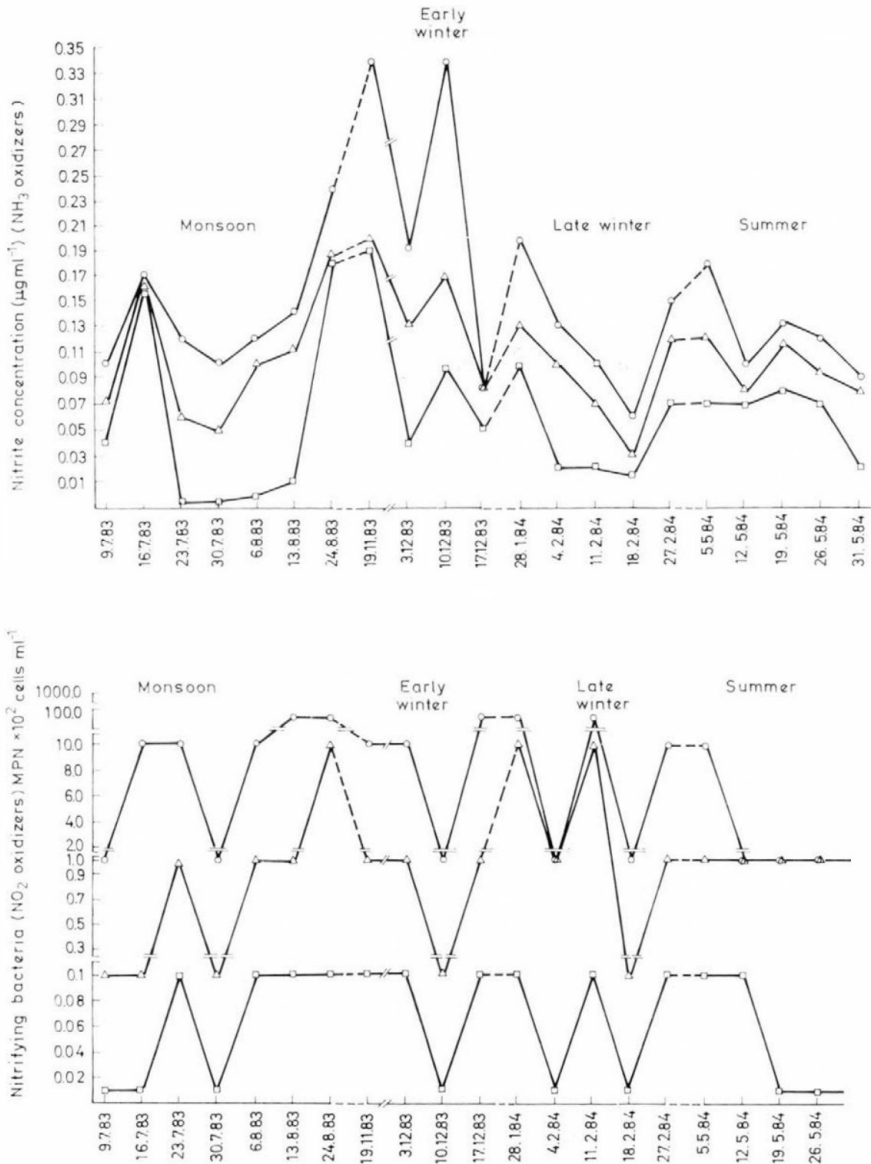


Fig. 2. Weekly changes of chemical concentration of produced nitrite (ammonia oxidizers) and nitrite oxidizers in four short term experiments. The trend of variation was very similar to that of long term ones. ○ Air breathing fish farming; △ polyculture; □ control

concentration of produced nitrite (ammonia oxidizers) and nitrite oxidizers in the short term studies (Fig. 2) were not different from the long term ones.

Quantification of bacteria. Quantification of data revealed that feed always harboured largest population of both ammonia oxidizers expressed in

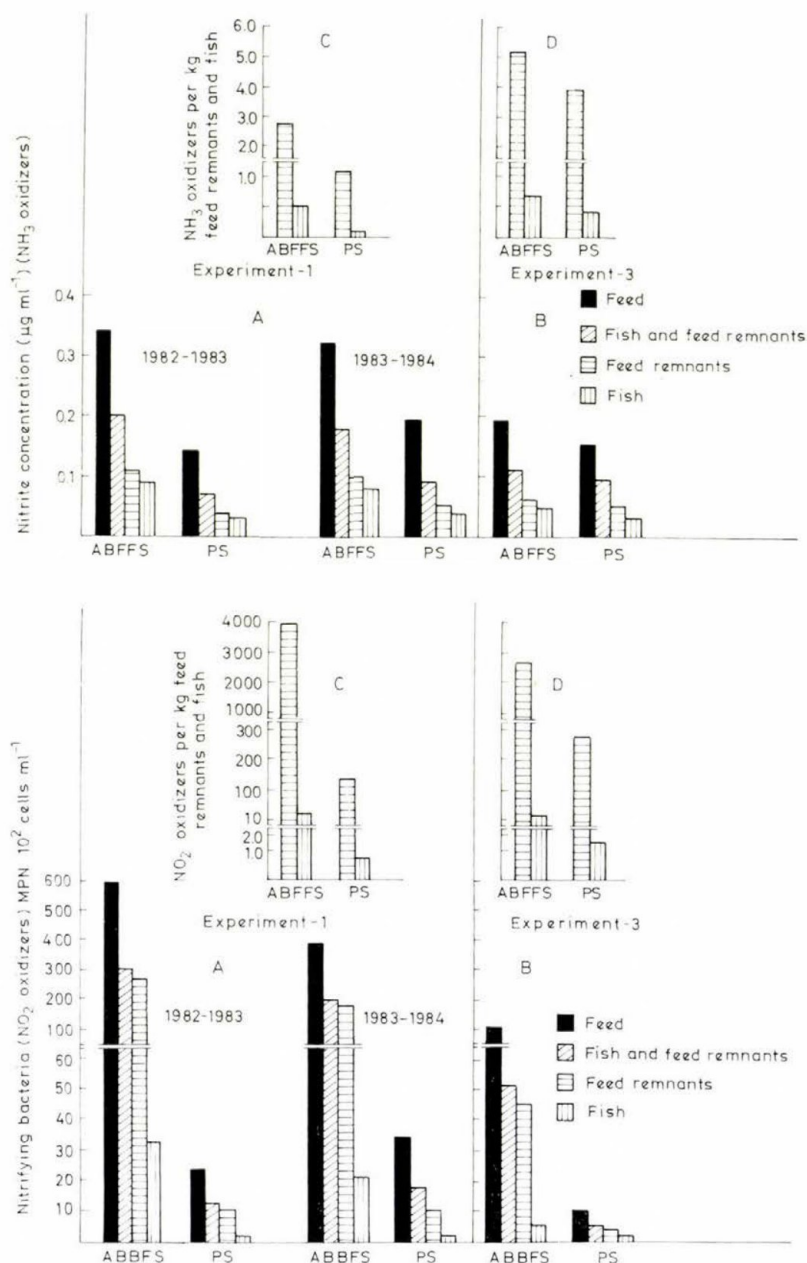


Fig. 3. Distribution pattern of the chemical concentration of produced nitrite (ammonia oxidizers) and density of nitrite oxidizers due to feed, feed remnants, fish metabolites. Application of feed resulted in maximum development of bacteria than any other segments of management protocols

terms of chemical concentration of produced nitrite (0.15 to $0.19 \mu\text{g ml}^{-1}$) and nitrite oxidizers (10 to $100 \times 10^2 \text{ cells ml}^{-1}$) either in the ABFFS or PS. The feed remnants of any two systems supported the next higher values of each of the two groups of bacteria. The metabolites of fish showed the lowest value of all. The load of bacteria observed in any part of ABFFS was at least 10% higher than that of PS (Fig. 3).

Discussion

It is evident that the distribution of the chemical concentration of produced nitrite (ammonia oxidizers) and the density of nitrite oxidizers were the functions of total nitrogen input in the system. Rearing of one kilogram of carnivorous air-breathing catfish in the rearing units resulted in the development of larger populations of ammonia oxidizers expressed in terms of chemical concentration of produced nitrite ($0.14 \mu\text{g ml}^{-2}$) and density of nitrite oxidizers expressed in terms of counts ml^{-1} ($14.9 \times 10^2 \text{ cells ml}^{-1}$) compared to the culture of herbivorous carps. Thus, even qualitative management protocols of ABFFS and PS were responsible for significant differences in both groups of nitrifying bacteria. This implied that the feed remnants and fish metabolites of carnivorous air-breathing catfishes caused greater eutrophication than herbivorous carps. It was calculated that one kilogram of feed remnants in 0.35 m^3 water volume generated the concentration of produced nitrite (ammonia oxidizers) to the tune of $5.1 \mu\text{g ml}^{-1}$ in the ABFFS and $4.0 \mu\text{g ml}^{-1}$ in the PS. The density of nitrite oxidizers was $26.9 \times 10^4 \text{ cells ml}^{-1}$ and $2.7 \times 10^4 \text{ cells ml}^{-1}$ in the ABFFS and PD, respectively.

The concentration of produced nitrite (ammonia oxidizers) in the PS and density of nitrite oxidizers in the ABFFS were significantly correlated ($r \geq 0.47$; $N=24$; $P < 0.05$) with the concentration of total nitrogen. Shilo and Rimon [7] showed that low level of ammonia did limit the multiplication of nitrifying bacteria in intensive fish farming ponds. In the present investigation, there was no clear cut relationship between the concentrations of ammonia-N and the chemical concentration of produced nitrite (ammonia oxidizers).

Though seasonal distribution of ammonia oxidizers in some polyculture ponds was highly correlated with dissolved oxygen [18], such a correlation either between the concentration of produced nitrite (ammonia oxidizers) or density of nitrite oxidizers and dissolved oxygen of tank water in the present study was not significant ($r \leq 0.33$; $N=24$; $P > 0.05$). This suggests that the dissolved oxygen was not necessary a condition for growth of nitrifying bacteria. Occurrence of nitrifying bacteria in anaerobic sediments was also reported by some investigators [19-21]. The distribution of chemical concentration of produced nitrite (ammonia oxidizers) and nitrite oxidizers in these

tanks was strongly affected by the ratio of total nitrogen to phosphate (≥ 0.39 ; $N=24$; $P < 0.05$).

Similar to the studies of Jana and Roy [18] for ammonia oxidizers, both ammonia and nitrite oxidizers in the present study exhibited a sharp peak during winter (23.3 °C) and decline in summer (30.5 °C). Although Niewolak and Korycka [22] and Rheinheimer [8] observed a sharp peak of nitrite bacteria during the summer months; the winter temperature of 23.3 °C observed in the present study was approximately close to the summer temperature of reported by the above investigators (20 to 25 °C). Moreover, *Nitrosomonas* is reported to be mesophilic type with a wide temperature tolerance range at 1–37 °C [23].

Acknowledgement. This research was supported by the University of Kalyani in the form of a Fellowship to SB.

REFERENCES

1. Guerin-Ancey, O.: *Aquaculture* **9**, 187 (1976).
2. Kaushik, S. J.: *Reprod Nutr Develop* **20**, 1757 (1980).
3. Colt, J., Armstrong, D.: Nitrogen toxicity to fish crustaceans and molluscs. Dept of Civil Engineering, University of California, Davis, California 1979. p. 25.
4. Russo, R., Thurston, R. V.: In Tubb, R. A. (ed.): *Recent Advances in Fish Toxicology*. EPA Ecology Research Series EPA-600/3-77-085. Environmental Protection Agency, Corvallis 1977. Pp. 118–131.
5. Diab, S., Shilo, M.: *Bamidgeh* **38**, 67 (1986).
6. Alexander, M.: *Introduction to Soil Microbiology*. Second Ed. Wiley Eastern Ltd., New Delhi 1978. p. 467.
7. Schmidt, E. L., Belser, L. W.: In *Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties*. Agronomy Monograph No. 9. Second Ed. American Society of Agronomy, Wisconsin 1982. pp. 1027–1042.
8. Rheinheimer, G.: *Aquatic Microbiology*. John Wiley, Chichester 1980. p. 235.
9. Gode, P., Overbeck, J.: *Z Allg Mikrobiologie* **12**, 567 (1972).
10. Stewart, W. D. P., Sinada, F., Christofi, N., Daft, M. J.: In Skinner, F. A., Shewan, J. M. (eds): *Aquatic Microbiology. Society for Applied Bacteriology, Technical Series No. 6*. Academic Press, London 1977. p. 369.
11. Witzel, K., Overbeck, J.: *Arch für Mikrobiologie* **122**, 137 (1979).
12. Barat, S., Jana, B. B.: *Bamidgeh* **39**, 120 (1987).
13. Lees, H., Quastel, J. H.: *Biochem* **40**, 806 (1946).
14. Skerman, V. B. D.: *A Guide to the Identification of the Genera of Bacteria*. Second Ed. Williams and Wilkins Co., Baltimore 1967. p. 2177.
15. Anon.: *Standard Methods for the Examination of Water and Wastewater*. Sixteenth Ed. American Public Health Association, Washington 1985. p. 1193.
16. Snedecor, G. W., Cochran, W. G.: *Statistical Methods*. Sixth Ed. Oxford and IBH Publishing Co., Calcutta 1967. p. 593.
17. Shilo, M., Rimón, A.: *Bamidgeh* **34**, 101 (1982).
18. Jana, B. B., Roy, S. K.: *J Appl Bact* **59**, 486 (1985).
19. Nikitina, N. S.: *Mikrobiologiya* **24**, 580 (1955).
20. Niewolak, S.: *Polish Arch Hydrobiol* **17**, 509 (1970).
21. Cavari, B. Z.: *Oikos* **28**, 285 (1977).
22. Niewolak, S., Korycka, A.: *Ekologia Polska* **27**, 625 (1979).
23. Wetzel, R. G.: *Limnology*. Second Ed. Saunders, Philadelphia 1983. p. 860.

RELATIVE SURFACE HYDROPHOBICITY, ANTIGEN K1 AND HAEMAGGLUTININATING ACTIVITY ARE ASSOCIATED IN *ESCHERICHIA COLI*

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(Received August 2, 1989)

Hydrophobic property of 136 *Escherichia coli* strains was examined by salt aggregation test (SAT). Out of the tested strains 61 were SAT positive. The correlations among the surface properties characterized by SAT and other phenotypical properties, e.g. mannose resistant haemagglutinating activity (MRHA), mannose sensitive haemagglutinating activity (MSHA), presence of antigen K1 and adsorption to $\text{Al}(\text{OH})_3$ gel were examined. The results showed that (i) Possession of antigen K1 provides the bacterial cell a hydrophilic character and covers its relative surface hydrophobicity; (ii) Correlation exists between the relative hydrophobicity of the bacteria determined by SAT and their haemagglutinating activity. SAT values are also influenced by non haemagglutinating fimbriae and also by other non fimbrial structures; (iii) The hydrophilic surface characters are mainly expressed by the results of adsorption to $\text{Al}(\text{OH})_3$ gel and the hydrophobic characters rather by the SAT values.

In recent years the studies concerning adhesins present on the surface of pathogenic bacteria and their role in colonization of mucous membranes have been in the centre of interest [1–10].

Although bacterial virulence is multifactorial [11] and there are different adaptation mechanisms, too [12], the adhesion of bacteria to host cells is a significant initial step in the pathomechanism of the infection.

There is a variety of mechanism by which the cells can bind to each other [4, 8, 13–15], therefore methods used to determine the surface character are of great importance.

For assessing the surface hydrophobicity of the bacterial cell different methods have been described [8, 10, 16–21].

We aimed to study the relative surface hydrophobicity of *E. coli* strains characterized previously by different pathogenic markers [22–25] by the modified salt aggregation test (SAT), which proved suitable for serial examinations. Furthermore, we wanted to compare former results of the $\text{Al}(\text{OH})_3$ gel adsorption test [26, 27] with the results of SAT.

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It is known that there is an association between the number and type of fimbriae and the hydrophobicity [9, 28] of a bacterial cell and also that strains possessing antigen K1 are characterized by negative surface charge [29]. We wanted to study the correlation between pathogenic markers (presence of antigens O and K, haemagglutination) and hydrophobicity of bacteria, to determine the connection between hydrophobicity obtained by SAT and other phenotypical properties.

Materials and methods

Bacterial strains. *E. coli* strains were isolated from faeces (healthy individuals, 6; patients with enteritis, 15; diagnosis unknown, 1), urine (pyelonephritis, 13; cystitis, 9; diagnosis unknown, 9), blood (10), cerebrospinal fluid (10) and from other extraintestinal sources (nose swab, 7; throat swab, 6; ear swab, 6; wound swab, 2; others, 12). The 15 K1 negative and the two K5 negative strains were obtained by the isolation of spontaneous phage resistant mutants from wild-type strains. The strains received from abroad or from other home institutes used in our work are listed in Table I [30–34]. The strains were stored at room temperature on Dorset egg medium (D) and stock agar medium (S) [35] and lyophilized.

Culture conditions. The strains were inoculated onto blood agar (BA), into broth (B) [35] and on colonization factor antigen (CFA) medium [36], and each medium was incubated at 18 °C, 37 °C and 42 °C, for 18 and 48 h.

Classification. The diagnostic criteria of the patients, the examination of O and H antigens [37], the detection of surface antigens K1 [38] and K5 [39], mannose resistant haemagglutination of human erythrocytes (MRHA) [40], the mannose sensitive haemagglutination of guinea pig erythrocytes (MSHA) [41], haemolysin production (Hly) [22] were described elsewhere [22–25, 27].

Gel adsorption. To determine the adsorptive capacity of the strains, the adsorption to $\text{Al}(\text{OH})_3$ gel was examined [26]. The AC_{50} value obtained by this method is the numerical value of the molarity of the phosphate buffer, which gives an adsorption equilibrium of 50% on $\text{Al}(\text{OH})_3$ gel.

Modified SAT method. The adsorption properties of the strains were also studied by the salt aggregation test (SAT) [9, 10, 42] modified by us. The "salt aggregation test" described by Lindahl et al. [10] was modified so that it could be carried out on glass slides. From the bacterial mass grown on solid media a poppy-seed sized amount, from liquid cultures a loopful amount was suspended in drops of $(\text{NH}_4)_2\text{SO}_4$ solution of different molarity on slides. Serial dilutions of phosphate buffered $(\text{NH}_4)_2\text{SO}_4$ varied at 0.02 M steps from 0.2 M to 1.8 M and at 0.2 M steps from 1.8 M to 3.2 M. The strength of the aggregation was evaluated as usual in slide agglutination [43]. The reaction was regarded as positive only (Figs 1, 2) when the aggregation was typical (the bacteria were aggregated into white clumps 0.1 mm in diameter and the background cleared up.) The reaction was regarded as atypical when the aggregate was rude and rough or a bit filamentous and the system did not clear up (Fig. 3). The SAT value was expressed as the lowest molarity of $(\text{NH}_4)_2\text{SO}_4$ which caused typical aggregation. Evaluating the results, those strains were regarded positive, which gave typical reactions at 2.0 M or at lower molarities. Dubious strains caused typical reaction between 3.2 M and 2.0 M and negative ones did not aggregate even above 3.2 M.

Results

Influence of environmental conditions on SAT values. In preliminary experiments it was found that the SAT values were influenced by different environmental effects. When the strains were stored on slant agar (SL) for 2 months the SAT value increased as compared to cultures stored for 18 h. After serial passage on BA, on B and on CFA medium, the SAT values remained at

Table I*Characters of strains received from institutes in Hungary and abroad*

Designation of strains	Origin of strains	Main characters of strains
AD110	J. F. van den Bosch [30]	F7 type strain; MRHA ⁺ ; Col ⁺ ; Hly ⁺
AD112	J. F. van den Bosch [30]	MSHA ⁺ ; Col ⁻ ; Hly ⁺
009	L. Emödy (Pécs, Hungary)	the strain was isolated from urine (of patient with haemolytic anaemia); MRHA ⁺ ; Hly ⁺
010	L. Emödy (Pécs, Hungary)	spontaneous Hly ⁻ mutant of strain 009, MRHA ⁺
536	J. Hacker et al. [31]	Hly ⁺ ; S fimbria ⁺ , serum resistance ⁺
536/21	J. Hacker et al. [31]	Hly ⁻ ; S fimbria ⁻
536/31	J. Hacker et al. [31]	Hly ⁻ ; S fimbria ⁺ , serum resistance ⁺
119	Kuch et al. [32]	isolated from urinary tract infection; MRHA ⁺ ; K5 ⁺ ; Hly ⁺
119/1	Kuch et al. [32]	spontaneous MRHA ⁻ , Hly ⁻ mutants of strain 119
H 10407	Evans et al. [33]	CFA I reference strain
H 10407 P ⁻	Evans et al. [33]	CFA I negative derivate of strain H 10407
PB 176	Evans et al. [34]	CFA II reference strain
PB 176 P ⁻	Evans et al. [34]	fimbrialess derivate of strain PB 176
895	H. Steinrück [GDR]	strain isolated from cerebrospinal fluid of patient with meningitis
896	H. Steinrück [GDR]	strain isolated from cerebrospinal fluid of patient with meningitis

MRHA = mannose resistant haemagglutination of human A group erythrocytes
 MSHA = mannose sensitive haemagglutination of guinea pig erythrocytes
 Col = colicinogenic property
 Hly = haemolysin production
 CFA I = colonization factor antigen I
 CFA II = colonization factor antigen II

Table II*Effect of culture conditions on the aggregation of bacteria*

Designation of strains	Storage of strains		Conditions of reaction							
	(S, 20 °C)		medium			temperature			pH of (NH ₄) ₂ SO ₄	
	18 h	2 months	BA	CFA	B	18 °C	37 °C	42 °C	5.2	6.8
H10407	0.12*	2.88	0.12	0.06	0.04	2.88	0.12	3.2	0.056	0.12
H10407 P ⁻	2.24	2.88	2.88	2.88	2.24	3.2	2.24	3.2	2.56	2.24
PB176	0.54	1.4	0.54	0.12	0.10	2.24	0.54	3.2	0.056	0.54
PB176 P ⁻	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2

S = slant agar; BA = blood agar; CFA = solid medium to demonstrate colonization factor antigen; B = broth

* SAT value: the numerical value of the lowest molaric concentration of the (NH₄)₂SO₄ solution at which typical aggregation was observed

For salt aggregation test the cultures were cultivated on blood agar at 37 °C for 18 h

the same level (Table II). The SAT values were also influenced by the quality of the medium: cultivating the strains on BA, CFA (37 °C, 18 h) and B (37 °C, 48 h) the SAT values increased in the following sequence: B → CFA → BA. The SAT values were higher, when the strains were incubated at 18 °C and 42 °C, respectively, compared to the results obtained after cultivation at 37 °C. The change was greater, when the temperature was 18 °C. The decrease of the pH (6.8) of the phosphate buffered $(\text{NH}_4)_2\text{SO}_4$ solution resulted in the decrease of the SAT values of some strains.

Correlation between SAT and the phenotypic characters of bacteria. A total of 136 *E. coli* strains of Hungarian and foreign origin was examined using the

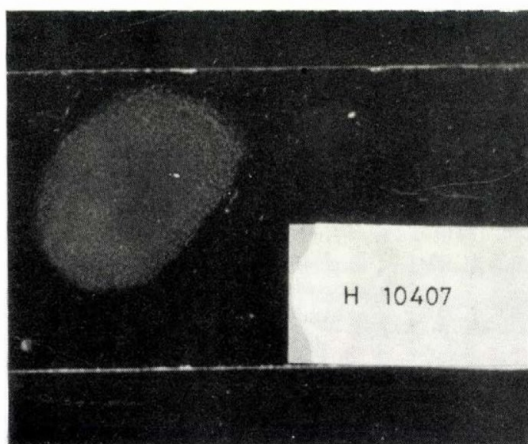


Fig. 1. Salt aggregation test of an 18 h blood agar culture of strain *E. coli* H 10407 in 0.12 M $(\text{NH}_4)_2\text{SO}_4$ solution. The reaction is typical, the background liquid is clear



Fig. 2. Salt aggregation test of an 18 h blood agar culture of strain *E. coli* 29166 in 0.96 M $(\text{NH}_4)_2\text{SO}_4$ solution. The reaction is typical, the background liquid is clear

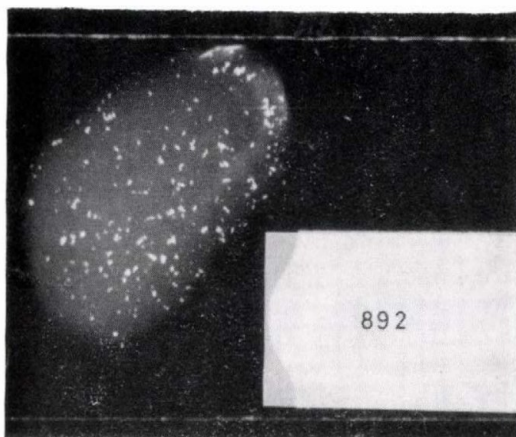


Fig. 3. Salt aggregation test of an 18 h blood agar culture of strain *E. coli* 892 in 1.92 M $(\text{NH}_4)_2\text{SO}_4$ solution. The reaction is atypical, coarse aggregated particles are suspended in a cloudy fluid

SAT method and 61 strains (43.3%) aggregated lower than at 2.0 M $(\text{NH}_4)_2\text{SO}_4$. Out of the aggregating strains 51 were MRHA positive.

The 106 home isolates classified previously into two AC_{50} groups [27] belonged also to two groups on the basis of SAT. Fifty-five strains belonged to group SAT I, they aggregated at higher concentration than 2.0 M (Table III) and the majority of the strains [41] aggregated at higher than 3.0 M. The AC_{50} values of the group varied between 0.01 and 0.04. Out of the 55 strains 51 possessed K1 surface antigen, 2 strains produced haemolysin and 20 were MRHA positive.

Out of the 51 strains which belonged to group SAT II 47 gave typical reaction at a lower concentration than 2.0 M (Table IV), whereas the SAT values of further 4 strains were not higher than 2.24 M. The AC_{50} values of the strains in this group varied from 0.001 to 0.009. K5 surface antigen was found in 18 strains, haemolysin was produced by 41 and 40 strains were MRHA positive.

Considering that surface antigen K1 was carried by the majority of the strains belonging to group I, experiments were carried out to find out, whether there is a correlation between the presence of antigen K1 and aggregation at high $(\text{NH}_4)_2\text{SO}_4$ concentrations. In these experiments 15 K1 positive strains and their spontaneous K1 negative mutants were involved. Table V shows that the SAT values decreased parallel with the loss of antigen K1.

The strains belonging to group II were characterized by frequent MRHA positivity, haemolysin production and when antigen K was carried, it was of type K5. To clear up the connection between hydrophobicity and the haemag-

Table III

Classifying of *E. coli* strains according to SAT values: group SAT I
Common character: AC₅₀0.01–0.04

No. of strains	Serotype	Phenotypic marker			SAT	
		K1	Hly	MRHA	>3.0 M	>2.24 M
4	O1 : H7	4	1	4	2	4
1	O1 : H34	1	—	—	—	1
1	O1 : HNT	1	—	1	—	1
5	O1 : H ⁻	5	—	3	3	5
1	O2 : H4	1	—	1	1	1
1	O2 : H6	1	1	—	—	1
7	O2 : H ⁻	7	—	2	4	7
1	O5 : H4	—	—	—	1	1
1	O5 : H ⁻	1	—	—	1	1
1	O7 : H4	—	—	—	1	1
1	O7 : H6	1	—	—	—	1
1	O7 : H7	1	—	1	1	1
7	O7 : H ⁻	7	—	5	5	7
1	O18ab : H ⁻	—	—	—	1	1
10	O18ac : H7	10	—	—	10	10
1	O21 : HNT	1	—	—	1	1
3	O45 : H7	3	—	3	2	3
1	O83 : H4	1	—	—	1	1
1	O83 : HNT	—	—	—	1	1
5	O83 : H ⁻	5	—	—	5	5
1	Sp : H ⁻	1	—	—	1	1
Total 55		51	2	20	41	55

AC₅₀ = numerical value of phosphate buffer molarity giving 50% adsorption equilibrium on Al(OH)₃ gel

K1 = surface antigen of type K1

Hly = haemolysin production

MRHA = mannose resistant haemagglutination of human group A erythrocytes

SAT = salt aggregation test; numerical value of the lowest molarity of (NH₄)₂SO₄ at which typical aggregation occurs

HNT = H antigen is not typable

Sp = spontaneously agglutinable

glutinating capacity of the strains, a representative group of strains was cultivated of fimbriae, according to literary data [44]. Furthermore, parallel examinations were carried out with MRHA positive and spontaneous MRHA negative mutants (119, 119/1). MRHA positive (AD110) and its MRHA negative/MSHA positive isogenic pair (AD112), Hly positive/Hly negative (009, 010) and the isogenic pair of strains 536 (Hly⁺, S fimbria⁺) 536/21 (Hly⁻, S fimbria⁻) 536/31 (Hly⁻, S fimbria⁺). The obtained values are presented in Table VI.

Table IV

Classifying of E. coli strains according to SAT values: group SAT II
Common character: AC_{50} 0.001–0.009

No. of strains	Serotype	Phenotypic marker			SAT <2.0 M
		K5	Hly	MRHA	
3	O2 : H1	—	3	1	3
3	O2 : H6	—	3	1	2
1	O2 : HNT	—	1	—	1
2	O2 : H ⁻	—	2	2	2
2	O4 : H1	—	2	2	2
7	O4 : H5	—	5	7	6
1	O4 : H ⁻	—	—	1	1
12	O6 : H1	6	12	12	12
1	O6 : H5	—	1	—	1
2	O6 : HNT	—	2	2	2
1	O6 : H ⁻	1	1	1	1
6	O18ac : H ⁻	6	5	6	5
1	O75 : H7	—	—	—	1
1	O75 : HNT	1	1	—	1
3	O75 : H ⁻	3	3	3	2
1	O78 : H ⁻	—	—	1	1
1	O86 : H9	—	—	—	1
1	O157 : H16	—	—	—	1
1	O157 : H38	—	—	1	1
1	O157 : H45	—	—	—	1
Total 51		17	41	40	47

Abbreviations: see Table III

Table V

Association between the presence of K1 surface antigen and mean SAT values of E. coli strains

No. of strains	K1 antigen	AC_{50}	Mean SAT values		
			BA	CFA	B
15	positive	0.012—0.048	> 3.2	1.28	1.28
15	negative	0.0012—0.0056	2.2	0.72	0.36

Abbreviations: see Tables II and III

It was found, that the SAT values of the strains cultivated at 18 °C and 42 °C (not seen on the Table), were higher, comparing to the values obtained at 37 °C and the number of haemagglutinating strains and the intensity of haemagglutination decreased parallel. This change was not reflected in the AC_{50} values.

Table VI
Connection between hydrophobicity and haemagglutinating capacity in E. coli strains

Designation of strains	18 °C				
	AC ₅₀	CFA		B	
		SAT	HA	SAT	HA
113	< 0.00125	> 3.2	—	> 3.2	—
117	0.0018	> 3.2	—	> 3.2	—
118	0.0032	> 3.2	—	> 3.2	—
121	0.0017	> 3.2	—	> 3.2	—
243	< 0.00125	> 3.2	—	> 3.2	—
467	0.0035	> 3.2	—	> 3.2	—
637	0.0019	> 3.2	—	2.24	—
1245	0.0018	> 3.2	—	> 3.2	—
119	•	> 3.2	—	3.2	—
119*	•	> 3.2	—	> 3.2	—
119/1	•	2.9	—	2.56	—
119/1**	•	2.9	—	3.2	—
AD 110	•	2.24	—	2.56	(MS)
AD 112	•	2.24	—	2.88	(MS)
009	•	2.54	—	2.56	(MS)
010	•	1.5	—	2.56	•
536	•	> 3.2	—	2.56	—
536/21	•	> 3.2	—	3.2	—
536/31	•	> 3.2	—	2.24	—
329***	•	> 3.2	—	> 3.2	—
330***	•	> 3.2	—	> 3.2	—

Designation of strains	37 °C				
	AC ₅₀	CFA		B	
		SAT	HA	SAT	HA
113	0.003	2.24	MR	2.88	—
117	0.0021	1.28	MR	0.72	MS
118	0.0037	1.28	MR	0.36	MS
121	0.0030	1.92	—	0.72	MS
243	0.0038	1.6	MR	0.36	MS
467	0.0038	2.88	MR	0.96	MS
637	0.0018	1.28	MR	0.54	MS
1245	0.0036	2.24	MR	0.36	MS
119	< 0.00125	1.6	MR	0.12	MS
119*	0.0021	2.88	MR	0.08	MS
119/1	< 0.00125	> 3.2	—	3.2	—
119/1**	< 0.00125	> 3.2	—	3.2	—
AD 110	•	0.36	MR	0.54	MS
AD 112	•	1.8	MR	0.36	MS
009	0.025	1.8	MR	0.36	MS
010	0.0018	1.8	MR	0.36	MS
536	0.007	> 3.2	—	2.54	MS
536/21	0.0125	> 3.2	—	> 3.2	—
536/31	0.007	> 3.2	—	2.24	MS
329***	0.0105	> 3.2	MR	3.2	MS
330***	0.013	> 3.2	MR	3.2	MS

Abbreviations: see Tables II and III

* K5 negative spontaneous mutant of strain 119

** K5 negative spontaneous mutant of strain 119/1

*** K1 surface antigen was carried by the strain

(MS) weak, late reaction

• not determined

Discussion

Conditions of the salt aggregation test. During the experiments the used strains were not freshly isolated. Faris et al. [9] described that their strains were stored at -70°C in 15% glycerol containing "Trypticase Soy Broth" or at $+4^{\circ}\text{C}$ in "Heart Infusion Agar". Our strains were stored on D (Dorset) and SL (slant-agar) medium at room temperature and lyophilized. For our experiments the majority of the strains were stored on D medium. In the course of the experiments the strains were kept on slant agar at room temperature. Table II shows that the SAT values of the strains stored on slant agar medium for 2 months at room temperature increased, i.e. their hydrophobicity decreased. No such changes were found among such storage conditions by O or K surface antigens or H antigens neither did the strains agglutinate spontaneously. The hydrophobicity of such strains did not increase after several passages. It may be assumed that the expression of components responsible for hydrophobicity is influenced by the adaptation to the environment. Accordingly, for such experiments only strains isolated freshly or stored under specific conditions can be used.

It is known from the literature, that the manifestation of the surface characteristics is influenced by the culture medium, too. For cultivation of the strains CFA [9, 10, 42] and BA medium [9], in case of strains isolated from faeces repeated subculturing in CFA broth are suggested [42]. The effect of cultivation on solid media (CFA, BA) and in liquid medium (B) on the modification of SAT values was examined. It was found, that the hydrophobicity of the bacterial surface increased if the strains were cultivated in static liquid medium. Probably in this case several membrane components of apolar character are manifested and a summation of their hydrophobic character occurs. Furthermore, it is known that the cells are more likely turned into fimbrial state in a static, liquid culture.

Similarly to the earlier observations of Lindahl et al. [10], examining part of the strains, it was found that the decrease of pH has an increasing effect on hydrophobicity. In other cases the change of the pH was not followed by a changes of the SAT value.

Aggregation is time-dependent: when the time of reaction is increasing, the value of the end-point decreases, while the frequency of the atypical reactions increases [10].

The concentration of bacterial cells was $5.10^9/\text{ml}$ in the standard SAT [10]. In the SAT method modified by us, the density of the suspension taken from the surface of the solid medium was similar to an 18 h broth culture (10^8 cells/ml).

Correlation between SAT and phenotypic characters of bacteria. It was found that out of the 53 K1 positive strains 43 were SAT negative, so they were

less hydrophobic (Tables III, V, VI). Out of the 15 K1⁺/K1⁻ isogenic pairs of strains the loss of K1 antigen resulted in an increase in hydrophobicity in 13 cases. The findings of Allen et al. [29] were similar, they compared the surface characters of K1 positive and K1 negative *E. coli* strains and they found that the K1 positive strains were characterized significantly by a higher negative surface charge.

We observed that the two groups, obtained on the basis of the adsorptive characteristics of the bacteria, differed from each other in respect of serogroups, too. According to Smyth et al. [8] the hydrophobicity of bacteria is independent of the O and K antigens. In the opinion of Magnusson et al. [45], who carried out experiments with S and R mutants, hydrophobicity is in connection only with the presence of the intact O antigens. In our opinion the simultaneous occurrence of certain serogroups and certain SAT groups is only an illusory connection between O antigen and hydrophobicity (Tables III and IV). It is known from the examinations of Czirók et al. [22-25] that there is a connection between serogroups O4, O6, O18 and haemolysin production and mannose resistant haemagglutination in *E. coli*. Because MRHA positivity is characteristic for group SAT II (in contrast with groups SAT I), the above grouping is connected with the haemagglutinating capacity and the associated O serogroups, besides the hydrophobic character of the strains. The problem is complicated by the fact that the opposing surface components (antigen K1 and haemagglutinating fimbria) many occur together, too. Out of 30 haemagglutinating but SAT negative strains 24 possessed antigen K1. It may be assumed that the bacterial cell is "masked" by antigen K1, i.e. the fimbriae responsible for hydrophobicity are covered by them, and so the hydrophilic character of the strains predominates. The hydrophobicity of strains possessing antigen K1 is not demonstrable by SAT.

The connection between haemagglutination and hydrophobicity is influenced by the manifestation of the proper membrane receptors of the bacterial cell. Ljungh et al. [42] observed that the bacteria were less fimbriated when cultured at 18 °C. According to our results, 13 out of the examined 21 strains were SAT positive (at 37 °C) and 11 gave mannose resistant haemagglutination with human erythrocytes, but none of the strains cultivated at 18 °C showed hydrophobic character and haemagglutination. Consequently, the loss of MR fimbriae resulted in the decrease of the hydrophobic character of the bacteria.

Because MR and MS fimbriae may be carried simultaneously by the strains [46] we examined the effect of the different fimbriae on hydrophobicity. The hydrophobicity determined on solid medium (CFA) was compared to the hydrophobicity determined in the sediment of 48 h broth culture (Table VI). In both cases the strains proved to be SAT positive, meaning that MS fimbriae are also responsible for hydrophobicity besides MR.

The strains were HA negative and SAT positive in 8.7%. In accordance with this result Ljungh et al. [42] observed that the non-haemagglutinating strains aggregated in 9.5%. It is obvious that the presence of fimbriae causing haemagglutination and the hydrophobicity of the strains are correlated. Though nonhaemagglutinating fimbriae and fimbriae of different antigenic type may be carried by the same bacterial cell [46], hydrophobicity may be caused by not fimbria-like proteins [47] there are not well known or not identified fimbriae [46, 48], MRHA and MSHA negative, but aggregating bacteria may occur.

The data presented in Tables III, IV, V and VI show that the character of the strains obtained by $\text{Al}(\text{OH})_3$ gel adsorption method are in correlation with the hydrophobicity of the strains determined by SAT. However, by the former method it is possible to distinguish differences between the "more hydrophilic" surface properties (when the SAT gives only values higher than 3.2 m), the SAT method is more sensitive for more hydrophobic surface properties.

The results of these experiments proved that the hydrophobicity of the bacterial surface can be determined by the SAT method. The SAT values are comparable with the values obtained with $\text{Al}(\text{OH})_3$ gel adsorption, but the SAT method among standardized condition is easier, quicker and cheaper. Because of its strict correlation with haemagglutination, we can draw conclusion not only to the adhesive capacity of the bacteria but also to those membrane components, which make possible this adhesion in vivo (MR, MS fimbriae).

The SAT method is based on in vitro experiments, it is advantageous to examine different materials and various cultivating conditions. The results should not be adapted directly to in vivo conditions. Our further aim is to support by in vivo experiments the role of hydrophobicity of bacteria in the interaction of cells and as consequence, in pathogenicity.

REFERENCES

1. Beachey, E. H.: *J Infect Dis* **143**, 325 (1981).
2. Duguid, J. P., Old, D. C.: In Beachey, E. H. (ed.): *Bacterial adherence*. Chapman and Hall, London 1980. p. 185.
3. Wadström, T., Faris, A., Freer, J., Habte, D., Hallberg, D., Ljungh, A.: *Scand J Infect Dis* **24**, 148 (1980).
4. Faris, A., Lindahl, M., Wadström, T.: *FEMS Microbiol Lett* **7**, 265 (1980).
5. Norlander, L., Norquist, A., Davies, J., Magnusson, K.-E., Normark, S.: *Scand J Infect Dis* **24**, 158 (1980).
6. Brinton, C. C.: In Miller, C. (ed.): *XIIIth U. S.-Japan Conference on Cholera*. Atlanta, Ga. 1977. p. 34.
7. Öhman, L., Hed, L., Stendahl, O.: *J Infect Dis* **146**, 751 (1982).
8. Smyth, C. J., Jonsson, P., Olsson, R., Söderlind, O., Rosengren, J., Hjertén, S., Wadström, T.: *Infect Immun* **22**, 462 (1978).
9. Faris, A., Wadström, T., Freer, J. H.: *Current Microbiology* **5**, 67 (1981).

10. Lindahl, M., Faris, A., Wadström, T., Hjertén, S.: *Biochim Biophys Acta* **677**, 471 (1981).
11. Ofek, I., Beachey, E. H.: In Beachey, E. H. (ed.): *Bacterial adherence*. Chapman and Hall, London 1980. p. 1.
12. Siverblatt, F. J.: *J Exp Med* **140**, 1696 (1974).
13. Derjagin, B. V., Landau, L. D.: *Acta Physicochimica USSR* **14**, 633 (1941).
14. Verwey, E. J., Overbeek, J. Th. G.: *Theory of the stability of liophobic colloids*. Elsevier, Amsterdam 1948.
15. Watt, P. J., Ward, M. E.: In Beachey, E. H. (ed.): *Bacterial adherence*. Chapman and Hall, London 1980. p.273.
16. Magnusson, K.-E., Johansson, G.: *FEMS Microbiol Lett* **2**, 225 (1977).
17. Van Oss, C. J.: *Ann Rev Microbiol* **32**, 19 (1978).
18. Kjelleberg, S., Lagercrantz, C., Larsson, Th.: *FEMS Microbiol Lett* **7**, 41 (1980).
19. Rosenberg, M., Gutnick, D., Rosenberg, E.: *FEMS Microbiol Lett* **9**, 29 (1980).
20. Rozgonyi, F., Szitha, K., Hjertén, S., Wadström, T.: *J Appl Bact* **59**, 451 (1985).
21. Mamo, W., Rozgonyi, F., Brown, A., Hjertén, S.: *J Appl Bact* **62**, 241 (1987).
22. Czirók, É., Marton, A., Csik, M., Solt, M.: *Acta Microbiol Hung* **31**, 187 (1984).
23. Czirók, É.: *Acta Microbiol Hung* **32**, 183 (1985).
24. Czirók, É., Milch, H., Csiszár, K., Csik, M.: *Acta Microbiol Hung* **33**, 69 (1986).
25. Czirók, É., Milch H., Vincze, I.: *Egészségtudomány* **30**, 217 (1986).
26. Szöllősy.: *Acta Microbiol Acad Sci Hung* **17**, 205 (1970).
27. Czirók, E., Petheő, G., Szöllősy, E., Milch, H., Herpay, M., Csik, M.: *Acta Microbiol Hung* **34**, 219 (1987).
28. Sherman, P. M., Houstin, W. L., Boedeker, E. C.: *Infect Immun* **49**, 797 (1985).
29. Allen, P. M., Roberts, I., Boulnois, G. J., Saunders, J. R., Hart, C. A.: *Infect Immun* **55**, 2662 (1987).
30. Van den Bosch, J. F., Postma, P., Graaf, J., Maclaren, D. M.: *J Med Microbiol* **14**, 321 (1981).
31. Hacker, J., Hughes, C., Hof, H., Goebel, W.: *Infect Immun* **42**, 57 (1983).
32. Kuch, B., Pál, T., Emődy, L.: *Acta Microbiol Acad Sci Hung* **27**, 317 (1980).
33. Evans, D. G., Silver, R. P., Evans, D. J. Jr., Chase, D. G., Gorbach, S. L.: *Infect Immun* **12**, 656 (1975).
34. Evans, D. G., Evans, D. J. Jr.: *Infect Immun* **21**, 638 (1978).
35. Bartha, T.: In Lányi, B. (ed.): *Public Health and Clinical Bacteriology. Standard Methods* (Hung.) National Institute of Hygiene, Budapest 1980.
36. Evans, D. G., Evans, D. J. Jr., Tjoa, W. S.: *Infect Immun* **18**, 330 (1977).
37. Ørskov, I., Ørskov, F.: *Med Microbiol Immunol* **99**, 163 (1977).
38. Gross, R. J., Cheasty, T., Rowe, B.: *J Clin Microbiol* **6**, 548 (1977).
39. Gupta, D. S., Jann, B., Schmidt, G., Golecki, J. R., Ørskov, I., Ørskov, F., Jann, K.: *FEMS Microbiol Lett* **14**, 75 (1982).
40. Evans, D. J. Jr., Evans, D. G., DuPont, H. L.: *Infect Immun* **23**, 336 (1979).
41. Evans, D. J. Jr., Evans, D. G., Young, L. S., Pitt, J.: *J Clin Microbiol* **12**, 235 (1980).
42. Ljungh, A., Wadström, T.: *Eur J Clin Microbiol* **1**, 388 (1982).
43. Lányi, B., Szita, J., Domján, J., Svidró, A.: In Lányi, B. (ed.): *Public Health and Clinical Bacteriology. Standard Methods* (Hung.). National Institute of Hygiene, Budapest 1980.
44. Ørskov, I., Ørskov, F., Sojka, W. J., Leach, J. M.: *Acta Pathol Microbiol Scand* **53**, 404 (1961).
45. Magnusson, K. E., Stendhal, O., Tagesson, C., Ebedo, L., Johannson, G.: *Acta Pathol Microbiol Scand* **85**, 212 (1977).
46. Duguid, J. P., Clegg, S., Wilson, M. J.: *J Med Microbiol* **12**, 213 (1979).
47. Kihlström, E.: *Scand J Infect Dis Suppl* **24**, 141 (1980).
48. Cravioto, A., Scotland, S. M., Rowe, B.: *Infect Immun* **36**, 189 (1982).

BINDING OF LECTINS (CON-A, LENS, HELIX, PHA) TO BINDING SITES INDUCED IN *TETRAHYMENA* BY INSULIN AND LECTINS

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(Received April 10, 1990)

Concanavalin-A (Con-A) gave rise to a moderate aggregation in untreated (control) *Tetrahymena pyriformis* GL populations. The cells pretreated (imprinted) with insulin or lectins underwent aggregation earlier and to a greater degree than the untreated control cells. Lens lectin, although of the same sugar specificity as Con-A, was considerably less active than the latter. Helix lectin and phytohaemagglutinin (PHA), although of similar sugar specificity, accounted for a dissimilar extent of cell aggregation, in that Helix lectin was comparable in activity to Con-A. Pretreatment (imprinting) with insulin enhanced the aggregation inducing effect of those lectins which were capable of overlapping on the insulin receptor owing to similar specificity.

The cytoplasmic membrane of *Tetrahymena* contains special structures, which are able to recognize the environmental molecules (signals) and to “remember” these at later interaction(s). A striking example of this phenomenon is the unicellular’s response to hormones of higher vertebrates, which give rise at primary interaction to hormonal imprinting and thereby to alterations in binding capacity and functional response, which persist over several hundred offspring generations of *Tetrahymena* [1–3].

The signal-recognizing membrane structures (receptors) being chemically glycoprotein-like compounds, their sugar components bind not only the adequate (hormone) molecule, but also certain lectins [4–7]. Thus like in mammalian cells, insulin and Concanavalin-A (Con-A) may overlap on the receptors of *Tetrahymena* [8–10].

In earlier studies along this line, the binding sites induced with insulin or Con-A accounted on reexposure to Con-A for a greater and more durable cell aggregation relative to the control [11]. This indicated that primary interaction gave rise to a quantitative increase in sugar-containing membrane structures and insulin Con-A overlap had taken place on the receptor.

In the present study we investigated the specificity of the binding site forming and recognizing capacity of lectins, as well as the extent of overlap of lectins of similar, or dissimilar sugar specificity on the membrane binding sites of *Tetrahymena*.

Materials and methods

Cultivation and treatment of Tetrahymena pyriformis GL. Cells maintained in 0.1% yeast extract containing 1% Tryptone (Oxoid, England) for 24 h at 28 °C were treated for 4 h with 10^{-6} M insulin (Semilente, MC, Novo, Denmark) or 1 mg/ml lectin (Con-A, phytohaemagglutinin, lens lectin or Helix lectin). Con-A was a product of Serva, FRG; the other lectins were prepared in this Institute by one of us, (P.K.). Untreated cells were used as control. After incubation the cells were washed thoroughly, returned to plain medium for 20 h, spread on slides in suspension, mixed with an equal volume of 10^{-6} M insulin or a 5 mg/ml lectin solution, and were observed for behaviour under the microscope for 3 min. Micrographs were taken during observation. Since no objective method was available for reading cell aggregation, five replica experiments were performed in each series.

Results

The experimental results, which were identical in each replica assay, are shown in Table I. The control cells responded by delayed, yet distinct aggregation exclusively to Con-A, and remained irresponsive to the other lectins.

Table I
Induction of cell aggregation by lectins in Tetrahymena cultures imprinted and not imprinted with insulin or lectins

Imprinter	Response on reexposure to			
	Con-A	Lens lectin	Helix lectin	PHA
—	+	?	—	—
Insulin	++	++	?	?
Con-A	++	—	++	?
Lens lectin	++	?	++	?
Helix lectin	++	—	+	+
PHA	++	?	++	?

+ Late onset

? weak uncertain response

The cells pretreated with insulin responded by a similar degree of aggregation to both Con-A and lens lectin.

Pretreatment with Con-A accounted for massive aggregation under the influence of both Con-A and Helix lectin, and a similar although somewhat lesser response was observed, after pretreatment with lens lectin or PHA.

The cells pretreated with Helix lectin responded to Con-A by marked aggregation and to Helix lectin and PHA by moderate aggregation.

Several lectins accounted for a minor temporary cell aggregation; this effect, indicated in Table I with question-marks, is not further discussed in this report, owing to its uncertainty.

Discussion

Of the ligands studied, Con-A developed the most powerful aggregating effect not only in pretreated but also in not pretreated (control) cultures. Less powerful, yet comparable effects were developed by Helix lectin, which differs from Con-A in sugar specificity, whereas lens lectin of similar sugar specificity to Con-A, had relatively negligible aggregating effect. Differences in the binding intensity of Con-A and lens lectin were also observed by other authors [10]. In this light, the powerful aggregating effect of Helix lectin on all lectin-pretreated cells could be explained by a possible relationship between the number of binding sites and degree of aggregation. It is known that while 6 binding sites are available for Helix lectin (one on each subunit), only 4 and 2, respectively, are available for Con-A and lens lectin [12, 13]. However, if the extent of aggregation depended exclusively on the number of binding sites, PHA, which also interacts with 4 binding sites, ought to have been exactly as active as Con-A, which was definitely not the case. Accordingly, factors other than the binding site number may also play a role in ligand-induced cell aggregation.

Lens lectin showed a 50 times lesser mannose activity than Con-A, a relatively greater haemagglutinating activity and a 10 times lesser inhibitory effect on the conjugation of *Tetrahymena* [10, 14]. These differences in behaviour were well portrayed in the present study by the dissimilar aggregating activity of the above two lectins. The dissimilar behaviour of Helix lectin and PHA was less easily explicable, because these bind to similar sugars. The similar aggregating effect of Con-A and Helix lectin on *Tetrahymena* cells pretreated with any lectin is also obscure, because the sugar specificity of these lectins is different. The problem seems to be still greater, if it is taken into consideration that Helix lectin aggregated the cells pretreated with insulin only for a transitory period, and failed to aggregate the control cells altogether. This supports the hypothesis that pretreatment with the lectins in question accounted not only for a quantitative increase in the specific sugar component of the membrane, but also for appearance of other sugar and amino sugar components. Since, however, this cannot fully explain the dissimilar behaviour of lectins of similar sugar specificity either, there is reason to believe that the nature of the interacting lectin, too, plays a role in the aggregating effect [15].

Present knowledge on the nature of the lectins permits the hypothesis that while the lectins destroy by cytotoxic effect the susceptible cells of a given population, the cells possessing *ab ovo* more numerous binding sites will proliferate and ultimately internalize and digest the lectin. Since in the present experiment the concentration used for primary exposure (imprinting) was five times lower than the one used for reexposure, the assumption lies close at hand that while primary exposure served for food intake, the second exposure ac-

counted for aggregation of the cells possessing great numbers of receptors. The role of food receptors acquires (still more) importance if it is taken into consideration that certain cells — *Tetrahymena* in the present case — may prefer given lectins (as food), and form accordingly additional food receptors for these. The sugar components capable of lectin binding will then appear in the food receptors [16]. Last but not least, the molecular mass and steric configuration of a given lectin may probably also play a role in the induction of binding sites, irrespective of the lectin's sugar specificity.

REFERENCES

1. Csaba, G.: Biol Rev (Cambridge) **55**, 47 (1980).
2. Csaba, G.: Int Rev Cytol **95**, 327 (1985).
3. Csaba, G.: Experientia **42**, 750 (1986).
4. Katzen, H. M., Soderman, D. D., Green, B. G.: Biochem Biophys Res Com **98**, 410 (1981).
5. Sandra, A., Leon, M. A., Przybilisky, R. J.: Endocrinology **105**, 391 (1979).
6. Sorimachi, K., Yashamura, Y.: Biochem Biophys Res Com **122**, 204 (1984).
7. Suzuki, T., Makino, H., Kanatsuka, A., Osegawa, M., Yoshida, S., Sakamoto, Y.: Metabolism **33**, 571 (1984).
8. Csaba, G., Kovács, P.: Cell Mol Biol **28**, 153 (1982).
9. Kovács, P., Csaba, G., László, V.: Acta Physiol Hung **64**, 19 (1984).
10. Pagliaro, L., Wolfe, J.: Exp Cell Res **168**, 138 (1987).
11. Csaba, G.: Microbios (1991). In press.
12. Alroy, J., Ucci, A. A., Pereira, M. E. A.: In DeLellis, R. A. (ed.): Advances in Immunocytochemistry. Masson, New York 1984.
13. Prokop, O., Uhlenbruck, G., Köhler, W.: Vox Sang **14**, 321 (1968).
14. Stein, M. D., Howard, I. K., Sage, H. J.: Arch Biochem Biophys **146**, 353 (1971).
15. Katzen, H. M., Vicario, P. P., Mumford, R. A., Green, B. G.: Biochemistry **20**, 5800 (1981).
16. Lenhoff, H. M.: In Muscatine, L., Lenhoff, H. M. (eds): Coelenterate biology. Academic Press, New York 1974.

RESISTANCE OF *ESCHERICHIA COLI* TO SOME ANTIBIOTICS AND BIOCIDES IN THE INTESTINAL BIOFILM OF MICE*

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(Received June 27, 1990)

An artificial monoflora of *Escherichia coli* in mice, as well as their autochthonous *E. coli*, exhibited enhanced resistance to streptomycin, chloramphenicol, sodium hypochlorite and silver nitrate. The level of resistance of the monoflora, which was 10–32 times higher than the in vitro determined Minimal Bactericidal Dose (MBCD), reached its maximum on the 7th–9th day after implantation. This latency is a requirement for the stabilization of the monoflora. Formaldehyde and carbenicillin were equally effective in the planktonic and in the biofilm mode of growth. In the case of carbenicillin the pieces of mouse colon contained about 60% of the dose used for exposure, in contrast to the 3% rate of streptomycin, showing the excellent penetrating ability of carbenicillin into the intestinal biofilm.

Investigating the pathomechanisms by which various enteric bacteria act, one cannot overlook the fact that the bowel is covered by a thick mucous layer. Therefore, our previous studies were directed on the interactions between enteric bacteria and mucus, especially its main component, the mucin. It was proved that the utilization of mucin is a general character of the enteric bacteria tested, independently of their pathogenic or non-pathogenic character [1]. The bacterial ligand in this binding is a surface protein, which is common among these organisms [2].

One of the techniques used in these studies was a mucin-minimal medium. Aged cultures, or mixed cultures showed phenomena leading us to the hypothesis of a “quasi biofilm” character. Of the above-mentioned phenomena an enhanced resistance to the killing effect of streptomycin was also observed [3]. In the present paper in vivo mouse experiments are summarized demonstrating enhanced resistance to antibiotics and biocidal agents.

Materials and methods

Strains. For establishing an *Escherichia coli* monoflora, a serologically not classified strain of *E. coli*, previously isolated from a healthy mouse, designated as “murine *E. coli*” was used. For the determination of the level of streptomycin and carbenicillin test strains

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* Supported by the Scientific Research Council, Hungarian Ministry of Health (I.2.10/333) and by OTKA (3/85/86)

E. coli No. 35034 and *Pseudomonas aeruginosa* No. 170006 (both from the Hungarian National Collection of Medical Bacteria) were used.

Media. Luria broth and LB agar were applied. For counting of the colony forming units (c.f.u.) of *E. coli*, Endo agar was used.

Antibiotics and biocides. Streptomycin sulphate (Medexport, USSR), chloramphenicol (Chlorocid, EGIS), carbenicillin (Karbenicillin-Na, Polfa), NaOCl (NaOCl 10% + NaOH 1%, Hydroxigen), argentum nitricum (Reanal) and liquor formaldehydi saponatus (with 15% formaldehyde and 10% ethanol content).

Animal experiments were carried out in an outbred strain of mouse (LATI, Gödöllő, Hungary).

Establishing E. coli monoflora. Mice treated orally with 50 mg doses of streptomycin on each of two consecutive days were maintained under sterile conditions (housed individually in sterile glass jars in a disinfected box and fed by autoclaved food). On the day following the streptomycin pretreatment the elimination of the aerobic bowel flora were checked. The implantation of the *E. coli* strain was performed by oral infection in a volume of 0.5 ml with about 10^7 /ml germs.

The in vitro determination of the Minimal Bactericidal Doses (MBCDs) of the different antibiotics and disinfectants was made in LB in a larger, later on in a narrow scale of their concentrations adding LB (planktonic) culture of the strain. After incubation at 37 °C for 3 h the growth was read and from the dilutions showing growth inhibition the determination of killing was performed by plating.

The in vivo determination of the MBCD values done in mice carrying *E. coli* monoflora or autochthonous bowel flora. The mice were sacrificed and about 1.5 cm long pieces of their colon starting from the cecum were removed. These pieces of mouse colon were cut longitudinally, the faecal content was superficially removed and were put into tubes containing different quantities of MBCD of the studied antibiotic or biocide. After 3 h incubation at 37 °C the pieces of colon were transferred into 10 ml portions of LB and then shaken vigorously in a Vortex apparatus (CSAV T5001/II, 30 W) for one min. Thereafter the samples were diluted and plated. After overnight incubation at 37 °C the colonies of the surviving bacteria were counted.

In some instances the resistance level was retested in the planktonic phase. The *E. coli* colony isolated from the highest positive dilution of the highest concentration, was subcultured in LB and tested against the used agent. This method was employed in the case of autochthonous *E. coli*. First the resistance level of the colonic *E. coli* of not pretreated mice was determined, thereafter the isolated strain was tested following the principle described above.

Determination of the penetration ability of carbenicillin into the colonic biofilm was carried out in mice carrying *E. coli* monoflora at the 10th day after implantation. Pieces of their pooled colonic bowels were incubated in 2 MBCDs of carbenicillin (as a control also in 2 MBCDs of streptomycin) at 37 °C for 3 h. Then the bowel pieces were rinsed in saline. After intensive Vortex-treatment in a ratio of 4 ml saline per g, chloroform was added to the liberated mucous fluid to kill the bacteria. After evaporation of chloroform the sample was frozen at -20 °C. This material, as well as the antibiotic containing supernatant of the exposed colon were diluted in LB and assayed with the test strains for carbenicillin and streptomycin content. Growth inhibition was read after 5 h incubation at 37 °C.

Results

1. MBCD values of antibiotics and biocides determined in vitro and in vivo

The in vitro MBCD of streptomycin for the "murine" strain of *E. coli* was 87.5 µg/ml (1 MBCD). Mice carrying this strain as a monoflora showed an increasingly enhanced resistance reaching its highest level (16–25 MBCDs — 1400–2187 µg/ml) between the 7th and 10th day of their carriership (Table I). The MBCD of chloramphenicol for this strain in its planktonic phase was 100 µg/ml. The same strain in a colonic biofilm showed a very marked resistance

reaching its peak on the 7th day of the carriership: 32 MBCDs (3200 µg/ml). The possibility that the enhanced resistance in vivo is a result of mutation is negligible because of the high c.f.u. values (10^5 – 10^7) even in the highest antibiotic concentration. For control, the resistance level in the planktonic phase was retested with an isolate from the highest concentration (32 MBCD) of chloramphenicol and from the highest dilution there of (10^{-5}). The result showed a resistance level between 97 and 195 µg/ml — corresponding to the preliminary in vitro sensitivity level (Table I).

Table I

In vivo killing effect of streptomycin and chloramphenicol on *E. coli* carried as a monoflora by mice

Time after implantation (days)	Streptomycin			Chloramphenicol	
	experiment No. 1 resistance (MBCD)	experiment No. 2 resistance (MBCD)	germ count (c.f.u.)	resistance (MBCD)	germ count (c.f.u.)
1	1	•		•	•
3	•	2	8×10^3	8	8.9×10^6
5	5	4	1.5×10^5 — 1.0×10^6	16	1.1×10^7
7	25	8	8.0×10^3 — 1.0×10^5	32*	1.1×10^6
9	25	•	2.9×10^4	•	•
10	•	16	1.8×10^5	32	5.3×10^7
12	25	•	2.0×10^5	32	1.1×10^7
17	•	16	2.0×10^5	•	•
20	25	•	1.4×10^5	•	•

Mice with the *E. coli* monoflora were killed, pieces of 1.5 cm of their colon were left to stand for 3 at 37 °C in a series of antibiotic dilutions containing multiples of in vitro determined MBCD values (streptomycin = 87.5 µg/ml, chloramphenicol = 100 µg/ml). Thereafter colonic pieces were put in 10 ml of LB, treated by a Vortex apparatus, diluted and plated

* Isolated colony of *E. coli* from the highest dilution in its planktonic phase. The level of resistance was found between 97 and 195 µg/ml, corresponding to the original in vitro level

Three disinfectants having different bactericidal mechanisms were chosen randomly for testing their effectiveness on the biofilm mode of growth. Sodium hypochlorite (in vitro MBCD 0.0066‰, calculated on the basis of its NaOCl content), silver nitrate (planktonic MBCD 2.5 mg/ml) and liquor formaldehydi saponatus (in vitro MBCD 0.0075‰ calculated on the basis of its formaldehyde content) were used (Table II). The minimal bactericidal effect on the *E. coli* colonic monoflora was considerably enhanced in cases of NaOCl and AgNO₃, reaching after implantation a maximum level of resistance against 10, or 16 MBCDs on the 9th, or 7th days, respectively (Table II). In contrast, formaldehyde had practically the same effectiveness in vitro and in vivo (Table II).

Table II*In vivo* killing effect of some disinfectants on *E. coli* carried as a monoflora by mice

Time after implantation (days)	Sodium hypochlorite		Silver nitrate		Liquor formaldehydi saponatus	
	resistance (MBCD)	germ count (c.f.u.)	resistance (MBCD)	germ count (c.f.u.)	resistance (MBCD)	germ count (c.f.u.)
2	3	2.0×10^8	2	2.0×10^4	1	3.7×10^4
5	6	6.0×10^3	4	3.3×10^6	1	1.2×10^4
7	8	6.8×10^6	16	2.0×10^5	1	2.1×10^5
9	10	2.0×10^7	16	1.0×10^7	2	1.8×10^5
Value of 1 MBCD in the plank- tonic phase (in vitro)	0.0066% (calculated as its NaOCl content)		2.5 µg/ml		0.0075% (calculated as its formaldehyde content)	

For the method see Table I

It is clear that both in cases of antibiotics and biocides the maximum level of resistance was reached between the 7th and 9th days after implantation. The simplest interpretation of this finding is that this period is necessary to the development of a colonic *E. coli* monoflora having all characteristics of a biofilm. A simple control of this assumption was carried out by sacrificing groups of monoflora carrying mice daily after implantation to determine the dynamics of the intestinal growth. The growth curve presented in Fig. 1 together with the standard deviation (2 SD) values among individual animals seems to be in good correlation with the curves of resistance changes. The c.f.u. values reached a plateau on the 7–9th days with lower SD values (Fig. 1). This may mean the “stabilization” of the artificial *E. coli* monoflora.

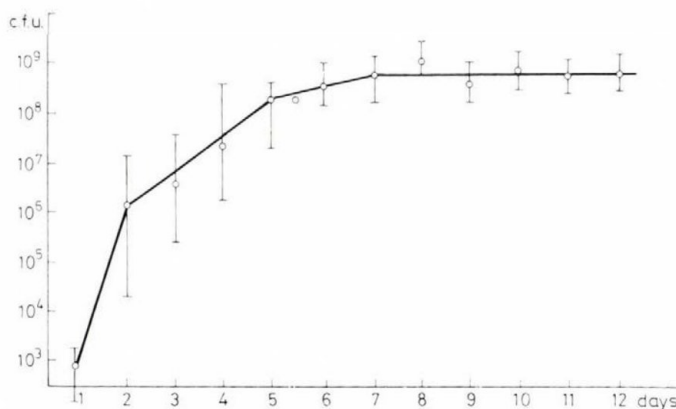


Fig. 1. Intestinal growth of the *E. coli* (monoflora). Groups of mice were sacrificed at daily intervals and their colonic pieces were used for c.f.u. determinations as described in Materials and methods

Table III
In vivo and in vitro sensitivity of the autochthonous E. coli of mice to bactericidal agents

Streptomycin				Chloramphenicol				Sodium hypochlorite			
in vivo		in vitro		in vivo		in vitro		in vivo		in vitro	
$\mu\text{g/ml}$	(c.f.u.)	$\mu\text{g/ml}$	(c.f.u.)	$\mu\text{g/ml}$	(c.f.u.)	$\mu\text{g/ml}$	(c.f.u.)	%	(c.f.u.)	%	(c.f.u.)
200	8.6×10^6	10	5.0×10^7	400	2.4×10^5	20	4.1×10^6	0.00012	1.4×10^8	0.00002	2.1×10^6
400	1.9×10^6	20	3.0×10^5	600	5.5×10^4	40	5.1×10^3	0.00025	1.6×10^5	0.00004	6.1×10^4
600	7.6×10^4	40	1.2×10^3	800	1.1×10^4	80	6.0×10^3	0.0005	3.9×10^4	0.00008	$< 10^3$
800	2.0×10^3	80	$< 10^3$	1000	0.6×10^3	160	$< 10^3$	0.001	$< 10^3$	0.00016	$< 10^3$
MBCD*	800 $\mu\text{g/ml}$	40 $\mu\text{g/ml}$		1000 $\mu\text{g/ml}$		80 $\mu\text{g/ml}$		0.001%		0.00008%	
Ratio**		20				12.5			12.5		

* Minimal Bactericidal Dosis

** Ratio of sensitivity between the biofilm/planktonic mode of growth

2. Investigations on the autochthonous intestinal *E. coli*

It is evident that our monoflora model is an artifact, since other components of the normal colonic flora may influence not only the number of *E. coli*, but also its resistance level to antibiotics or biocides. A simple experimental approach is to investigate the resistance level of the autochthonous *E. coli* in pieces of colon, followed by isolating *E. coli* from the highest concentration of the given bactericidal agent, assuming that a predominant survivor clone of *E. coli* has been recovered. Finally, the resistance level of this isolate in its planktonic phase is to be tested.

In this series of experiments streptomycin, chloramphenicol and sodium hypochlorite were used as bactericidal agents. The results are summarized in Table III. It is clear that the autochthonous *E. coli* of mice has also an enhanced resistance in the biofilm. In the case of streptomycin the rate of resistance levels between planktonic and biofilm mode of growth reached a value of 20, in cases of chloramphenicol and sodium hypochlorite of 12.5 (Table III). The results demonstrate that the observed phenomenon exists under natural environmental conditions, too.

3. The bactericidal effect of carbenicillin

Out of the biocides formaldehyde exerted a bactericidal effectiveness very similar in vitro and in vivo. An analogous finding for carbenicillin was investigated in detail. One MBCD of carbenicillin determined in vitro (250 µg/ml) was also effective on the biofilm mode of growth in the colonic mucus involving *E. coli* monoflora, as well as the autochthonous member of *E. coli* in the normal intestinal flora (Table IV).

Table IV

The effect of carbenicillin in the planktonic phase and in the biofilm mode of growth of E. coli representing the artificial monoflora and the autochthonous E. coli of mice — in vivo and in vitro

Time after implantation (days)	<i>E. coli</i> monoflora		Autochthonous <i>E. coli</i> resistance (MBCD)**	
	resistance (MBCD)*	germ count (c.f.u.)	in biofilm	planktonic
3	1	1.1×10^4		
5	<2	(●)	~1	~1
7	1	2.2×10^5		
10	1	1.5×10^7		

For explanation see Tables I and III

* 1 MBCD determined in vitro, 250 µg/ml

** 1 MBCD in case of the isolated strain, 125 µg/ml

Table V

The quantity of carbenicillin and streptomycin taken up by the pooled colonic pieces of mice carrying monoflora an exposure to 2 MBCDs

	Carbenicillin	Streptomycin
Quantity of antibiotics (2 MBCDs) used for exposure, μg	1600	550
Quantity of antibiotics taken up by colonic pieces (800 mg), μg	960	16
Ratio of antibiotics taken up	60%	2.9%
Test strains and MIC values	<i>P. aeruginosa</i> HNCMB 170006 10 $\mu\text{g}/\text{ml}$	<i>E. coli</i> HNCMB 35034 4 $\mu\text{g}/\text{ml}$

Groups of mice carrying the monoflora of *E. coli* "murine" strain were sacrificed, 1.5 cm long pieces weighing 800 mg of the colon starting from the cecum were collected and exposed to 2 MBCDs of carbenicillin or streptomycin. After 3 h incubation at 37 °C the pieces of bowel were treated by a Vortex apparatus after rinsing in saline. The mucous material was treated by chloroform and after evaporation frozen at -20 °C. The antibiotic content of them was determined by using sensitive standard test strains on the basis of growth inhibition read after 5 h incubation at 37 °C. The sensitivity of the test strains was determined earlier

According to Costerton et al. [4] the microbial resistance in biofilm may be caused by the altered metabolism — or by the reduced penetrating ability into this biofilm of the agent. This latter possibility seems to be easier to prove or disprove in a concrete case. Therefore we exposed pooled colonic pieces of mice carrying *E. coli* monoflora to low (2) multiple MBCDs of carbenicillin and, as a control, of streptomycin and after incubation the quantity of antibiotics taken up by the colonic mucus layer was determined. The samples of such exposed pieces were vortex-treated after rinsing in saline and their antibiotic contents determined by sensitive test strains. The results are summarized in Table V. Taking also into consideration the inaccuracy of this biological assay, it is evident that a clear cut difference exists between the uptake of carbenicillin and streptomycin: in case of carbenicillin about 60% of the exposed quantity was in the mucous fraction prepared from the colons, while this rate of uptake concerning streptomycin was about 3% (Table V). It seems that the excellent penetrating ability of carbenicillin into the intestinal biofilm may be a sufficient interpretation of the observed effect.

Discussion

It is remarkable that regarding the biological functions of the normal bowel flora, the very first and most favoured topic of microbiologists was the antagonistic role of the intestinal biofilm against enteric bacterial pathogens. Streptomycin treated *E. coli* free mice of Bohnhoff et al. [5] or of Peter [6]

showed an enhanced sensitivity against salmonella infections. We found that this difference of sensitivity was $2 \log_{10}$ exponent expressed in LD_{50} values. Furthermore, the reconstitution of the original level of the natural resistance is succeeded by an *E. coli* monoflora, but only after a length of a week's carriership [7]. Freter [8, 9] demonstrated a long lasting carriership of *Shigella flexneri* and *Vibrio cholerae* in mice and in guinea pigs after the elimination of the *E. coli* bowel flora by streptomycin. We were successful in repeating Freter's results: when using a selected virulent clone of *S. flexneri* 2a, the infective dose 50% (LD_{50}) was found to be 3×10^6 in the presence of the normal intestinal flora and 5×10^0 germs in its absence [10]. This antagonism is exerted not only against enteric pathogens: a similar phenomenon was observed between two *E. coli* strains [11]. Always the *E. coli* introduced first was able to establish a stable monoflora, while the secondary strain was eliminated after a short period of time.

These data are in an agreement with the conception of biofilm summarized at first by Costerton et al. [12] and specified for the intestines by Freter et al. [13] and Rosee et al. [14]. The biofilm involves also an elevated resistance against phagocytic cells, antibodies, antibiotics and biocides. Our experiments presented in this paper clearly demonstrate this enhanced resistance. The *E. coli* monoflora carried by mice shows a resistance against streptomycin and chloramphenicol which is about 25–32 times higher than the resistance level of the planktonic phase of the same strain. Among biocides the same phenomenon can be observed in cases of sodium hypochlorite and silver nitrate, with an enhancement of 10–16 times.

The methodological difficulties to enumerate bacteria from biofilms should also be mentioned. Costerton et al. [4] recommend beside a vortex-like intensive shaking also an ultrasonic treatment to liberate bacteria from the biofilm. Vortex-treatment only was also sufficiently effective. The remaining vortex-treated pieces of mouse colon yield only a few colonies when plated directly on media.

Beside the enhanced resistance, a stepwise elevation was also observed reaching the top-level on the 7th–9th day after implantation. The simple experiment shown in Fig. 1 indicates that this time dependency is caused by the slow intestinal growth of the introduced *E. coli* parallel with the formation of the "mature" biofilm. This "stabilization" actually happens between the 7th and 9th days. This is in a good correlation with our in vitro "quasi biofilm" experiments [3], with the cited [7] need of one week for the *E. coli* monoflora to reach the original resistance against salmonella infection and perhaps also with the finding of Davis et al. [15] that seven and a half day is necessary in suckling mice for the development a stable *E. coli* flora.

The artificial *E. coli* monoflora beside many advantages, e.g. no other factors of bacterial origin is influencing the characteristics of the single species

to be investigated, has a basic disadvantage: it is not a normal formation. Our experiments carried out on the autochthonous *E. coli* show that its enhanced resistance is not influenced by the fact that it is the only member of the intestinal flora.

Out of the few biocides tested, formaldehyde, and out of the antibiotics, carbenicillin were equally effective on the planktonic and on the biofilm mode of growth of *E. coli*. The carbenicillin effect was analysed only. Its equal effectiveness was demonstrated also on *E. coli* present either as a monoflora, or as a member of the normal flora. Furthermore, beside the inaccuracies of the applied biological assays it is clear that carbenicillin penetrates excellently into the colonic biofilm. Costerton et al. [4] noted the paradoxon, that our antibiotics were selected and tested only on the basis of their effect in a planktonic mode of growth. Antibiotics effective also in biofilms are badly needed in numerous problem of diseases. Our results for carbenicillin proved that such antibiotics can be found in our present regimen of drugs. Further work is necessary to reinvestigate the drugs used in this respect and perhaps it is also necessary to introduce some method(s) for elucidating the eventual effectiveness when developing new drugs.

REFERENCES

1. Kétyi, I.: Acta Microbiol Hung **35**, 389 (1988).
2. Kétyi, I.: Acta Microbiol Hung **37**, 45 (1990).
3. Kétyi, I.: Acta Microbiol Hung **36**, 41 (1989).
4. Costerton, J. W., Cheng, K.-J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., Marrie, T. J.: Ann Rev Microbiol **41**, 435 (1987).
5. Bohnhoff, M., Miller, C. P.: Proc Soc Exp Biol Med **86**, 132 (1954).
6. Peter, A.: Zentralbl Bakteriell (A) **182** 473 (1961).
7. Rauss, K., Kétyi, I., Hegyi, P.: Zentralbl Bakteriell (A) **190**, 360 (1963).
8. Freter, R.: J Exp Med **104**, 411 (1956).
9. Freter, R.: J Infect Dis **97**, 57 (1955).
10. Rauss, K., Kétyi, I., Angyal, T.: Path Microbiol **29**, 95 (1966).
11. Rauss, K., Kétyi, I.: Zentralbl Bakteriell (A) **177**, 161 (1960).
12. Costerton, J. W., Irwin, R. T., Cheng, K.-J.: Ann Rev Microbiol **35**, 299 (1981).
13. Freter, R., O'Brien, P. C. M., Macsai, M. S.: Infect Immun **34**, 234 (1981).
14. Rose, K. R., Cooper, D., Lam, K., Costerton, J. W.: Appl Environ Microbiol **43**, 1451 (1982).
15. Davis, C. P., McAllister, J. S., Savage, D. C.: Infect Immun **7**, 666 (1973).

A MATHEMATICAL METHOD FOR THE IDENTIFICATION OF THE ATGCAT RECOGNITION SEQUENCE OF A *STREPTOCOCCUS* *MUTANS* RESTRICTION ENDONUCLEASE

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(Received June 29, 1990)

Taking advantage of a mathematical equation applied so far in different fields the calculation of ATGCAT recognition sequence of restriction endonuclease from *Streptococcus mutans* serotype C (SmuC I) is reported together with confirming computer and physical mapping data.

A new restriction endonuclease has been isolated from *Streptococcus mutans* serotype C — SmuC I — (Geck et al. unpublished, patent pending) and for the identification of the recognition sequence a novel mathematical method has been used. The prediction of the number of cleavage sites in different restriction endonuclease-DNA reactions are mathematically established in the calculation of genetic diversity and sequence relatedness [1–5]. The probable number of sites can be calculated from the data of DNAs and restriction endonucleases by the exponential equation of Nei and Li [1], which was originally developed and used only in molecular genetics so far.

We attempted to extend the use of the equation in the opposite direction: cleavage sites were experimentally identified, total base pair and GC content data of substrate DNAs were available, GC content and nucleotide composition of the recognition sequence could be calculated and then confirmed by computer and enzymatic physical mapping.

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Materials and methods

Chemicals. Most of the chemicals used were from Reanal, Hungary and of analytical grade. The ethidium bromide and agarose (Type I, low EEO) were purchased from Sigma.

DNA substrates. Lambda phage DNA (48502 base pairs [6], GC content is 0.49 [7]) was purchased from Reanal. Adenovirus type 2 (35937 bp. [6], GC = 0.55 [7]) and adenovirus type 1 DNA (about 36000 pb. according to data in this laboratory, GC = 0.585 [7]) were given by Dr. P. Medveczky. The SV40 DNA (5243 bp. [6], GC = 0.406 according to computer calculations in this laboratory) was prepared as described by Geck and Nász [8] in form of an SV40-pBR322 recombinant plasmid obtained from Dr. Gy. Berencsi.

Restriction endonucleases. The restriction endonucleases were purchased from Amersham and Reanal and used in conditions specified by Sambrook et al. [9]. The SmuC I was prepared in this laboratory employing our purification method [10].

Agarose gel electrophoresis. A horizontal electrophoresis system was used with submerged gel, the DNA fragments were run in 1% agarose. The buffer and voltage were the same as in [11]. The DNA fragments were visualized by ethidium bromide fluorescence.

Results and discussion

The recognition sequence is a hexanucleotide. The equation of Nei and Li is as follows:

$$n = m_t [g/2]^{r_1} [(1-g)/2]^{r_2}$$

n = number of sites on the DNA

m_t = total nucleotide pairs of DNA

g = the GC content of the DNA

r_1 = the number of guanines and cytosines in the recognition sequence

r_2 = the number of adenines and thymines in the recognition sequence

The computer assisted calculations were carried out on adenovirus type 2 and lambda DNAs; 5, 6 or 7 recognized nucleotides were assumed containing 2, 4 or 6 palindromic GC in each case. The probable nucleotide number of the recognition sequence was the value giving the calculated cleavage site numbers in the range of real figures. Only six nucleotides with 2, 4 or 6 GC met this requirement on both adenovirus DNA (6.9, 10.4 and 15.5 sites, respectively — to the real 9) and lambda DNA (12.3, 11.3 and 10.4 sites, respectively — to

Table
GC content calculation

GC content	SV40			Adenovirus type 1		
	number of sites		diversity (%)	number of sites		diversity (%)
	calculated	real		calculated	real	
0	3.59	3	19.6	2.87	9	68.1
2	1.68	3	44	5.7	9	36.6
4	0.78	3	74	11.34	9	25.5
6	0.36	3	88	22.5	9	150

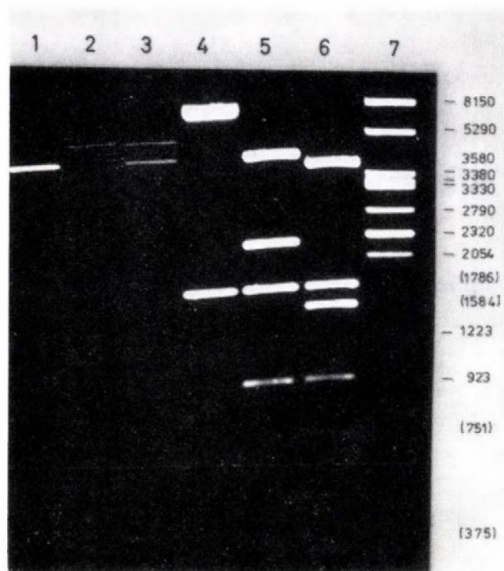


Fig. 1. Agarose gel electrophoresis: DNA fragments were visualized by ethidium bromide fluorescence. Lane 1: pBR322 DNA linearized by BamH I. Lane 2: SV40 DNA linearized by BamH I. Lane 3: the recombinant plasmid (SV40 cloned in BamH I site of pBR322) cleaved by BamH I. Lane 4: SV40-pBR322 recombinant cleaved by SmuC I and BamH I. Lane 5: SV40-pBR322 recombinant cleaved by SmuC I. Lane 6: SV40-pBR322 recombinant cleaved by SmuC I, BamH I and EcoR I. Lane 7: Molecular weight marker — adenovirus type 1 DNA digested by Hind III. The length of fragments are given in base pairs, figures in parenthesis refer to fragment in lane 6 (the 375 bp. fragment in lane 6 was cut from pBR322)

the real 14). Figures obtained with five recognized nucleotides (with 2 or 4 GC — adenovirus DNA: 31 and 46.2 sites; lambda DNA: 48.2 and 44.5 sites, respectively) or with seven nucleotides (containing 2, 4 or 6 GC — adenovirus DNA: 1.5, 2.3 and 3.5 sites; lambda DNA: 3.1, 2.9 and 2.6 sites, respectively) were far from the actual numbers of sites.

The hexanucleotide contains two GC. The theoretically expected number of sites on SV40, adenovirus type 1, type 2 and lambda DNA are shown in Table

I

in the recognition sequence

Adenovirus type 2			Lambda			Average diversity (%)
number of sites		diversity (%)	number of sites		diversity (%)	
calculated	real		calculated	real		
4.6	9		49	13.3		
6.96	9	22.6	12.3	14	5	35.4
10.4	9	15.6	11.36	14	12.2	28.8
15.54	9	72	10.44	14	18.9	33.5
					25.4	83.9

I, with zero, two, four and six GC in the hexanucleotide recognition sequence. The differences of calculated values from real figures were expressed in percents of the real values and the lowest average percentage of diversity (last column) was supposed to indicate the most probable GC content. On this rationale, two GC could be assumed in the recognition sequence, because the calculations produced the lowest value with two GC (28.8%), while with zero, four and six GC the average diversity percents were higher (35.4, 33.5 and 83.9, respectively).

ATGCAT is the probable recognition sequence. Owing to the use of the equation the further analysis could be focused on the possible 24 hexanucleotide with two GC and by the exclusion of those having sites on pBR322, this number could be reduced to even 12. Fortunately, of these twelve only one had three site on SV40 DNA: the ATGCAT sequence.

ATGCAT is confirmed by enzymatic and computer mapping. The enzymatic map of SmuC I sites on SV40 DNA (cloned in the BamH I site of pBR322) and the computer-localized ATGCAT sites on the complete nucleotide sequence of SV40 DNA [12] were compared.

SmuC I cleaved an about 1700 bp. fragment and a minor fragment (less than 100 bp., undetectable in agarose), while the rest of SV40 DNA was left in fusion with the total pBR322 (about 7500 bp.), as shown in Fig. 1. Lane 4 BamH I cut the SV40 pieces from pBR322 (about 2300 and 1000 bp.), indicating the distances of SmuC I sites from the BamH I site at 2451th bp. on SV40 DNA (Fig. 1, lane 5) and the EcoR I cleavage of the 2300 bp. fragment (at 1700th bp. position) localized the SmuC I sites at about the 150th and 3500th bp. (± 100 bp.) and the third is close proximity of one of these two (Fig. 1, lane 6). The computer-localized ATGCAT sites (at 61th, 116th and 3501th nucleotides) were in identical arrangement, confirming the result of mathematical analysis.

REFERENCES

1. Nei, M., Li, W. H.: *Proc Nat Acad Sci USA* **76**, 5269 (1979).
2. Gotoh, O., Hayashi, J. I., Yonekawa, H., Tagashira, Y.: *J Mol Evol* **14**, 301 (1979).
3. Kaplan, N., Langley, C. H.: *J Mol Evol* **13**, 295 (1979).
4. Tajima, F., Nei, M.: *J Mol Evol* **18**, 115 (1982).
5. Wijsman, E. M.: *Nucl Acid Res* **12**, 9209 (1984).
6. New England Biolabs, 1988-89 Catalog, pp. 118-125.
7. Mandel, M.: In Fasman, G. D. (ed.): *CRC Handbook of Biochemistry and Molecular Biology*. 3rd ed. Nucleic Acids Vol. I. CRC Press, 1975. pp. 549-564.
8. Geck, P., Nász, I.: *Anal Biochem* **135**, 264 (1983).
9. Sambrook, P., Fritsch, E. F., Maniatis, T.: *Molecular Cloning — A Laboratory Manual I-II-III*. 2nd ed., Cold Spring Harbor Laboratories Press, Cold Spring Harbor 1989. pp. 5.3-5.32.
10. Geck, P., Molnár, A., Nász, I.: *Acta Microbiol Hung* **34**, 241 (1987).
11. Helling, R. B., Goodman, H. M., Boyer, H. W.: *J Virol* **14**, 1235 (1974).
12. Reddy, W. B., Thimmappaya, B., Dahr, R., Subramanian, K. H., Zain, B. S., Pan, J., Ghosh, P. K., Celma, M. L., Weisman, S. M.: *Science* **200**, 494 (1978).

IDENTIFICATION OF ATGCAT SEQUENCE AT SITES OF SMUC I RESTRICTION ENDONUCLEASE BY COMPUTER AND PHYSICAL MAPPING OF ADENOVIRUS TYPE 1 DNA

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(Received July 26, 1990)

Physical mapping of adenovirus type 1 DNA was carried out in order to analyze the recognition sequence of a novel *Streptococcus* restriction endonuclease. In addition to the new map and homology data on this poorly analyzed serotype, the result offers the definite evidence for the ATGCAT recognition sequence on adenovirus DNA and the physical map of cleavage points.

The resistance of *Streptococcus* to transformation by different DNAs has long been observed, and a search for the reason resulted in the identification of a novel restriction endonuclease, SmuC I (Geck et al. unpublished, patent pending). A hexanucleotide recognition sequence of this enzyme could be assumed by computer calculations (for mathematics see [1]), based on the number of sites identified in this laboratory on different DNAs (lambda phage DNA — 14, adenovirus type 2 DNA — 9, SV40 DNA — 3, pBR322 plasmid DNA — 0).

Since the enzyme had no site on pBR322 plasmid DNA, the palindromic hexanucleotides present on the nucleotide sequence of this DNA [2] could be excluded by computer analysis, thus the number of the possible nucleotide combinations decreased considerably.

The full presentation of foregoing experiments is beyond the scope of this paper, but prior to going into details, a short summary of early results is given here for completeness and clarity, mentioning questionable points as well.

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Briefly, the locations of the possible hexanucleotides were identified on the nucleotide sequence of SV40 DNA [3], and these computer constructed maps were compared with the enzymatic physical map. Only ATGCAT sequence could be related to all three cleavage sites. These preliminary data, however, seemed to be insufficient for a definite result, because different, possibly non-palindromic or degenerated sequences accidentally locating near the identified sites could not unequivocally be excluded by these very few sites analyzed. Theoretically, experiments in the same fashion with a sufficiently big number of sites on a substrate DNA of known nucleotide sequence could definitely decide the recognition sequence. Adenovirus type 2 DNA meets these requirements and the identification of the ATGCAT recognition sequence applying the above strategy on adenovirus DNA is reported in this paper.

Materials and methods

Chemicals. Most of the chemicals used were of analytical grade and from Reanal, Hungary. The ethidium bromide and agarose (Type I, low EEO) were purchased from Sigma, USA.

DNA substrates. Adenovirus type 1 and type 2 DNAs were generous gifts from Dr. P. Medveczky. For the easy analysis of results presented in this paper the positions (in base pairs) of cleavage sites of restriction endonucleases used in the analysis of adenovirus DNA are given in consecutive order from left to right on the DNA of adenovirus type 1 [4, 5]:

EcoR I: 27300, 30100

Sal I: 9450, 9800, 16700, 29500

BamH I: 15400, 21600, 32400

Hind III: 2800, 6200, 11600, 13600, 15100, 18300, 26300, 28700, 32600, 34900

The total number of base pairs of adenovirus type 2 DNA is 35937 bp. (base pairs) [6], and a similar figure (about 36000 bp.) was obtained for adenovirus type 1 DNA according to data in this laboratory.

Restriction endonucleases. The restriction endonucleases were purchased from Reanal, Hungary, Amersham, UK and used in conditions specified by Sambrook et al. [7]. The SmuC I restriction endonuclease was prepared by a procedure established in this laboratory (Geck et al. patent pending, unpublished).

Agarose gel electrophoresis. A horizontal electrophoresis system was used with submerged gel, the DNA fragments were run in 1% agarose. The buffer and voltage were the same as in [8]. The DNA fragments were visualized by ethidium bromide fluorescence.

Results and discussion

Sequence homology in the region of interest on adenovirus type 1 and type 2 DNA. Since adenovirus type 1 has been under investigation in this laboratory for years, we were interested in the physical map of SmuC I sites on this DNA, because the recognition sequence of the new enzyme was also an intriguing problem, both questions were tried to be answered in combination. The strategy outlined above for the recognition sequence analysis necessitates, however, the exact knowledge of the nucleotide sequence, but no available data concerning the sequence of adenovirus type 1 DNA could be collected.



Fig. 1. The fragment patterns of the SmuC I digestions of adenovirus type 1 and type 2 DNAs are shown in slots indicated. In the adenovirus type 2 pattern fragment B runs together with fragment C. Two additional very short fragments were detected in polyacrylamide gel (not seen in this agarose gel)

The nucleotide sequence of only adenovirus type 2 DNA has been identified and published so far either in parts [9], or in total (cited in [6]), so the adaptation of these data was attempted to the analysis of the left sixty — seventy percents of type 1 DNA, which is the region of homology according to several evidences. Adenovirus type 1 is the closest relative of type 2, belonging to the same subgenus (subgenus C) of adenovirus serotypes [10], and most of the restriction endonucleases tested cleave similar or identical fragment patterns from the two DNAs [10, 11], where the invariable sites locate at the left part of the genomes, while differences appear mostly at the right terminal thirty-fourty percents.

In Fig. 1 the fragments cleaved by SmuC I from type 1 and type 2 DNAs are shown. The patterns are completely identical, except fragment B, the right terminal fragment as later shown, locating in the region carrying variable sequences. The identical fragments refer to identical nucleotide sequences in rest of the two genomes, so the reasonable use of adenovirus type 2 nucleotide sequence data in case of this part of type 1 DNA seemed to be justified.

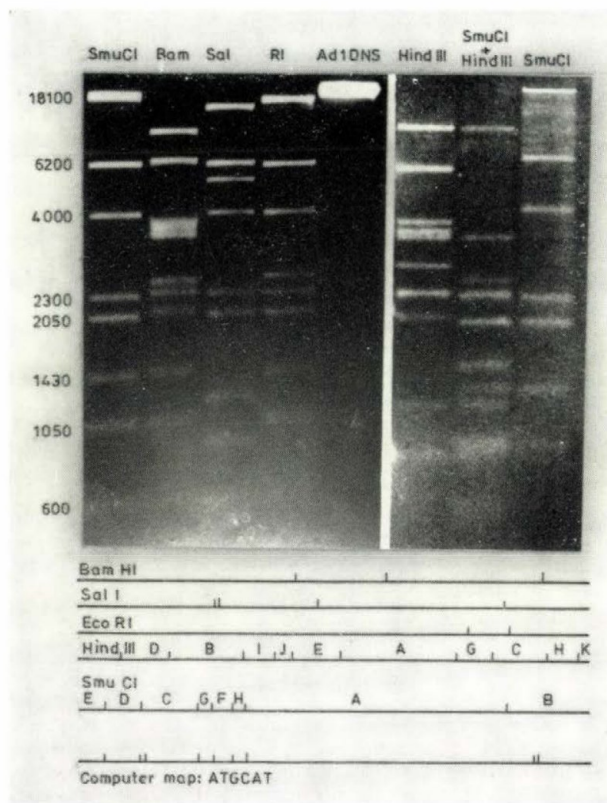


Fig. 2. In the left panel the fragment patterns of adenovirus type 1 DNA digested first with SmuC I and then with BamH I, Sal I and EcoR I enzymes are shown in slots indicated. In the right panel the fragment patterns of adenovirus type 1 DNA digested with Hind III and only with SmuC I are shown in slots indicated. In the bottom of the figure physical maps of the above enzymes are demonstrated together with the computer map of ATGCAT sites

Physical mapping of SmuC I sites on adenovirus type 1 DNA. The result of double digestions of adenovirus type 1 DNA are shown in Fig. 2. The original fragment pattern cleaved by SmuC I is shown in the first slot, the length of fragments are given in base pairs at the left side of the gel. These fragments were then digested by BamH I, Sal I, EcoR I and Hind III restriction endonucleases and the results are demonstrated in the slots indicated. In case of Hind III an original adenovirus type 1 DNA–Hind III digestion is also shown to demonstrate fragment differences. For the easy analysis of fragment changes and construction of physical map the cleavage points of the above enzyme are given at the bottom of the figure and positions of sites in Materials and methods.

The result of SmuC I–EcoR I double digestion can the most easily be interpreted. Nearly all fragments remained intact, only fragment A and fragment B moved slightly faster and a new, about 2600 bp. fragment appeared above fragment D. Since the novel fragment is about as long as the shortest fragment cleaved by EcoR I (see EcoR I map), this fragment could only be cut from fragment A, because from other fragments this loss would have led to a drastic mobility shift. A small piece is also cleaved from fragment B causing a very slight mobility shift and means, that A and B are neighbouring fragments and so these fragments can not be placed anywhere else, just as fragment B the right terminal and fragment A is next to B.

In SmuC I — Sal I double digestion fragment A was cut shorter, fragments F and G are also shorter with about 150–200 bp. and a new, about 5100 bp. fragment appeared below fragment B. Obviously the latter originated from fragment A supporting the previously identified position of this fragment. In the Sal I map a double site is seen about 450 bp. apart and the mobility shifts of the small fragment F and G can only be explained, if these two are neighbouring fragments and their boundary lies between the double Sal I sites, so short pieces are cut from both upon digestion with Sal I. Fragment F must be placed at the right side of G according to base-pair calculations: the distance from the right end of fragment F to the middle Sal I site is 5700 bp. and though no fragment like this is present in this slot, difference between this and the newly appeared 5100 bp. fragment is 600 bp. — exactly the length of fragment H, so fragment H can be placed by this indirect evidence between fragment F and A. The sequence of fragments identified thus far is as follows: G — F — H — A — B (right terminal fragment).

No new information can be obtained from the SmuC I–BamH I double digestion, but the above results are confirmed in every aspect. The original fragment A is cut even shorter (about 8300 bp.), the original fragment B disappeared, but a bit longer novel fragment (about 6300 bp.) appeared nearly in its position and three more new fragments appeared between fragment C and D, with about 3800, 3500, 2500 bp. length. The only interpretation of these results is as follow. The 3800 bp. and the 6300 bp. fragments were cut from fragment A. The distance from the right side of fragment F to the left terminal BamH I site is about 4400 bp. This fragment is not seen in this slot, but the difference between this and the newly appeared 3800 bp. fragment is 600 bp. the length of fragment H, so a novel evidence is offered that it must be placed between F and A. The identified position of fragment B is also confirmed by the right terminal BamH I site, since it cleaves fragment B for two pieces and they appear in the calculated sites: the 3500 bp. and the 2500 bp. fragments.

In addition to data confirming the above results, the SmuC I–Hind III double digestion is helpful in sorting out fragments C, D and E. Since the Hind III fragment F (left terminal) is cleaved, the SmuC I left terminal frag-

ment can not be fragment C because it is longer, so only fragments D or E are possible. Fragment E was shown to be the left terminal by the results of incomplete digestion (data not shown), because the firstly appearing fragments of the complete pattern are always the terminal ones (since for their cleavage one cut is enough, while internal fragments need two cuts) and in this case fragments E and B appeared first, even after the shortest digestions. In localization of fragments D and C the strong, double, about 1600 bp. fragments in the double digestion are important, since they can only be cleaved from Hind III fragment D, when the left second SmuC I site halves this fragment, consequently SmuC I fragment C must be placed between fragments D and G according to calculations of lengths of possible fragments. Thus the final, complete sequence of fragments is as follows: E — D — C — G — F — H — A — B.

The next positions of SmuC I sites on adenovirus type 1 DNA have been identified (the approximate base pair positions are indicated from left to right and also expressed in percents of the total genom in parenthesis: 2050 (5.7%); 85 (23.4%); 9600 (26.46%); 11000 (30.64%); 11600 (32.3%); 29700 (82.7%). The map is visualized at the bottom of Fig. 2 and the length of fragments are also indicated in base pairs.

Comparison of the computer localized ATGCAT sites and the physical map. The computer data of ATGCAT sites on the adenovirus type 2 DNA were used either as a result of computer analysis carried out in this laboratory on published sequences of adenovirus type 2 DNA [9], or according to data in [6]. ATGCAT sequences were identified at the following positions: 2068, 4405, 4583, 8567, 9609, 11040, 11613, 31904, 31955. This map is also demonstrated in Fig. 2. Two double sites have been revealed by the computer (at 4405–4583 bp. and 31904–31955 bp. positions), but these very small fragments could not be identified by the method of agarose gel electrophoresis. The only discrepancy of the two maps is the difference in the position of the right terminal site, but since it is in the variable region, it refers to species differences.

The rest of sites are in completely identical position in both maps. These data, together with the result of the analysis of SV40 DNA as mentioned, convincingly demonstrate, that ATGCAT sequence plays a role in the recognition of SmuC I restriction endonuclease and so it is an isoschizomer of Ava III and Nsi I restriction enzymes. According to data in [12], Ava III and Nsi I have exactly the number of sites on lambda DNA, adenovirus type 2 DNA, SV40 DNA and pBR322 DNA which were obtained on the same DNAs by SmuC I digestion (see Introduction) offering a strong additional evidence for the ATGCAT recognition sequence.

REFERENCES

1. Nei, M., Li, W. H.: *Proc Natl Acad Sci USA* **76**, 5269 (1979).
2. Sutcliffe, J. G.: *Cold Spring Harb Symp Quant Biol* **43**, 77 (1979).
3. Reddy, W. B., Thimmappaya, B., Dhar, R., Subramanian, K. N., Zain, B. S., Pan, J., Geos, P. K., Celma, M. L., Weisman, S. M.: *Science* **200**, 494 (1978).
4. Perencsi, Gy., Medveczky, P., Naroditsky, B. S., Chaplygina, N. M., Zavizion, B. A., Tikhonenko, T. I., Nász, I.: *Acta Microbiol Acad Sci Hung* **25**, 97 (1978).
5. Medveczky, P., Zavizion, B. A., Perencsi, Gy., Chaplygina, N. M., Szomolányi, E., Naroditsky, B. S., Nász, I., Tikhonenko, T. I.: *Arch Virol* **67**, 85 (1981).
6. *New England Biolabs 1988-89 Catalog* pp. 124-125.
7. Sambrook, J., Fritsch, E. F., Maniatis, T.: *In Molecular Cloning — A Laboratory Manual I-II-III*, Cold Spring Harbor Laboratories Press, Cold Spring Harbor 1989, pp. 5.3-5.32.
8. Helling, R. B., Goodman, H. M., Boyer, H. W.: *J Virol* **14**, 1235 (1974).
9. Gingeras, T. R., Sciaky, D., Gilenas, R. E., Bing-Dong, J., Yen Ce, Kelly, M M., Bullock, P. A., Parsons, B. L., O'Neill, K. E., Roberts, R. J.: *J Biol Chem* **257**, 13475 (1982).
10. Adrian, Th., Wadell, G., Hierholzer, J. C., Wigand, R.: *Arch Virol* **91**, 277 (1986).
11. Tooze, J.: *Molecular Biology of DNA tumor viruses*. Appendix. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York 1980.
12. Kessler, C., Neumaier, T. S., Wolf, W.: *Gene* **33**, 1 (1985).

PURIFICATION OF A NEW RESTRICTION ENDONUCLEASE FROM *STREPTOCOCCUS MUTANS* AND IDENTIFICATION OF ITS RECOGNITION SEQUENCE

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SmuE I, a type II restriction endonuclease, has been isolated from *Streptococcus mutans* serotype E, which is an isoschizomer of *Ava II* recognizes the palindromic pentanucleotide sequence 5' GG/W/CC 3'. Similarly to *Ava II*, *SmuE I* cleaves the sequence, G^AG/W/CC, generating 5' protruding fragment termini.

Literary data [1, 2] and our own examinations of the resistance of *Streptococcus mutans* strains to transformation by different DNAs have led us to describe a new site specific restriction endonuclease, *SmuC I* from serotype C (Geck et al. unpublished results). Further experiments on other serotypes resulted in the isolation of a new restriction endonuclease, *SmuE I* — an isoschizomer of *Ava II* [3] — which recognizes the pentanucleotide sequence G^AG/W/CC, generating 5' cohesive termini, same as the *Ava II* [4].

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The great number of isoschizomers of *Ava II* suggested that we should be satisfied with only the purification step which allowed us to determine the precise recognition sequence and the main properties of *SmuE I*.

In the nomenclature of the restriction endonuclease the acronymic system of Smith and Nathans [5] has been followed.

Materials and methods

Chemicals. Most of the chemicals used were of analytical grade (Reanal, Hungary). Sepharose 6B (Pharmacia) and Whatman P11 Cellulose Phosphate were used. Ethidium bromide, agarose (Type I, low EEO) were purchased from Sigma, acrylamide and bisacrylamide were from Serva.

DNA substrates and nucleotides. Lambda phage DNA was purchased from Biotechnika Co. (Hungary), pUC19, and T7 phage DNAs were generous gifts from G. Mikala (Second Department of Biochemistry, Semmelweis University Medical School, Budapest), adenovirus type 2 DNA was from P. Medveczky (Department of Microbiology and Immunology, University of South Florida, Tampa, Fla, USA), pKL300 plasmid DNA a kind gift from A. Simoncsits (Institute of Genetics, Biological Research Center, Szeged, Hungary). The (α^{32} P)dATP was purchased from Amersham.

Enzymes. Restriction endonucleases were purchased from Amersham, DNA Polymerase I Large fragment (Klenow fragment) was from Bohringer, Mannheim, FRG, RNase (bovine pancreas) was from Reanal and the reaction conditions were according to Sambrook et al. [6].

Agarose gel electrophoresis. Horizontal electrophoresis system was used with submerged gel, DNA fragments were run in 1% agarose. The buffer and voltage were same as it was described earlier [7].

Bacterial strains and cultivation. The *Streptococcus mutans* serotype E strains was a kind gift from M. Orsós (Department of Microbiology, Semmelweis University Medical School, Budapest). The bacterial cells were cultivated in two litres Bouillon (Human, Hungary) medium supplemented with Yeast Extract (Merck) and at Tryptone (Oxoid) and were harvested in the late logarithmic growth phase (Janetzky K 23, 3500 rpm, 20 min, 4 °C) determined spectrophotometrically at 550 nm. At 37 °C cultivation lasted for about 10 h without shaking. Cells were washed in 10 mM Tris HCl pH 8.0, 1 mM EDTA and the wet bacterial mass was stored at -20 °C.

Results

Preparation of the crude extract. Bacterial cells were suspended in two volumes of extraction buffer: 20 mM Tris HCl pH 8, 7 mM 2-mercaptoethanol, 5 mM EDTA, 200 μ g/ml RNase. For disruption of cells an ultrasonic disintegrator was used (maximum output in short bursts, 5 min at 0 °C) and then Triton X-100 was added to final concentration of 0.2%. Cell debris was sedimented by ultracentrifugation (Janetzky VAC 601, 35000 rpm, 1 h, 4 °C). The supernatant was further purified by addition of activated phosphocellulose powder in a batch procedure for 20 min at 4 °C. The supernatant, obtained after pelleting the phosphocellulose by centrifugation (Janetzky K 23, 4000 rpm, 10 min, 4 °C) was immediately applied to gel filtration chromatography.

Gel filtration chromatography. Gel filtration chromatography was carried out on Sepharose 6B column (2 cm² × 1.5 m, eluent: 20 mM Tris HCl pH 8.0, 7 mM 2-mercaptoethanol, 5 mM EDTA buffer, at a flow rate of 0.1 ml min⁻¹

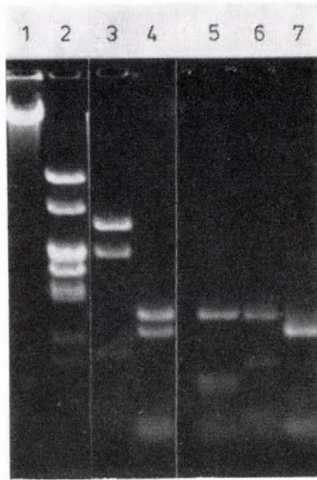


Fig. 1. Double digestion experiments. Lane 1: uncut adenovirus type 2 DNA. Lane 2: adenovirus type 2 DNA + Hind III — molecular weight marker. Lane 3: linearized pBR322 (4363 bp.) + pBR322 digested with Hinc II (A: 3256 bp. B: 1107 bp.) — molecular weight marker. Lane 4: pBR322 + SmuE I. (A ≈ 1750 bp., B ≈ 1450 bp., C/D/E/F ≈ 200–320 bp.). Lane 5: pBR322 + Hind III + Smu I — fragment A is intact, fragment B is divided for two smaller fragments (≈ 750–800 bp. and ≈ 630–680 bp.). Lane 6: pBR322 + BamH I + SmuE I — fragment A is also intact, fragment B is divided for a bigger and a smaller fragment (≈ 940–980 bp. and 430–480 bp.). Lane 7: pBR322 + Pvu II + SmuE I — fragment B is intact, fragment A is severed for a bigger fragment which run with fragment B and a smaller fragment which comigrate with fragment C

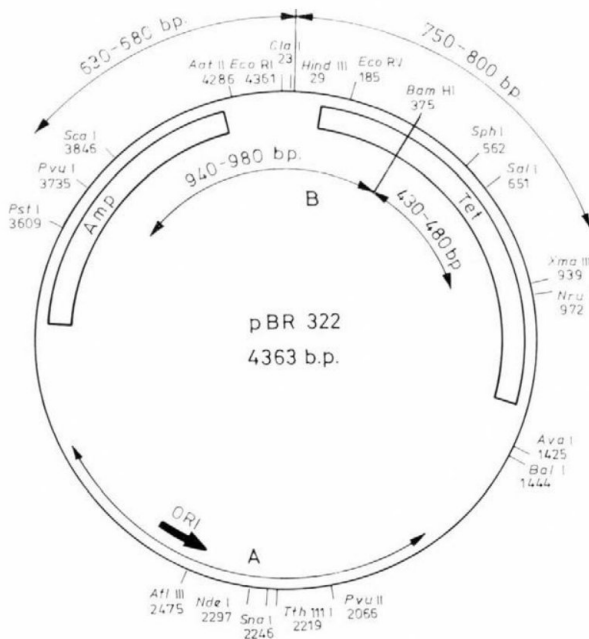


Fig. 2. The possible position of fragment A and B on pBR322 DNA are indicated

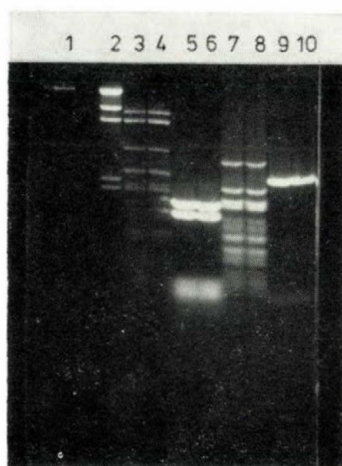


Fig. 3. The parallel digestion panel. Lane 1: uncut lambda phage DNA. Lane 2: lambda phage DNA + Hind III — molecular weight marker. Lanes 3, 5, 7, 9: lambda phage-, pBR322 plasmid-, T7 phage- and pUC19 plasmid DNA + SmuE I, respectively. Lanes 4, 6, 8, 10: lambda phage-, pBR322 plasmid-, T7 phage- and pUC19 plasmid DNA + Ava II, respectively

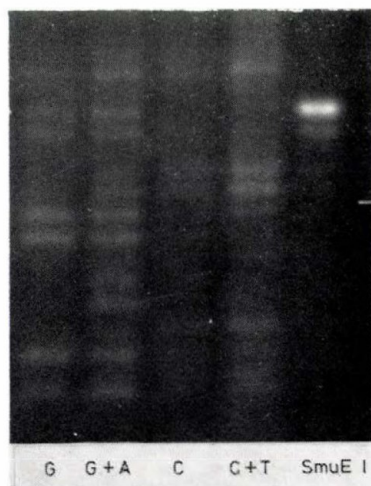


Fig. 4. The result of the sequencing. The SmuE I fragment comigrate with the first guanine nucleotide of the recognition sequence, which means that its length is shorter with one nucleotide (chemical sequencing), so the precise cleavage structure is 5' G^A G/W/CC 3'

[8]). Two ml fractions were collected and tested directly for the restriction endonuclease activity (data not shown). At this step the purity of the enzyme was clear enough to determine its recognition sequence.

The recognition sequence of SmuE I. The determination of recognition sequence of the new enzyme was carried out by digestion of pBR322 DNA with

SmuE I, which cleaves the plasmid DNA for more than five fragments. Taking advantage of double digestion experiments with SmuE I–Hind III, SmuE I–BamH I and SmuE I–Pvu II on pBR322 DNA (Fig. 1) allowed us to define the sizes and positions of the “A” and “B” fragments (Fig. 2). The possible recognition sequences on the ends of fragment “B” (50 bases before and after of the calculated end points) were analyzed by a computer program which predicted that the probable cleavage site is GG/A/T/CC similarly to the recognition site of Ava II. This identity was confirmed by parallel digestion on commonly used DNA molecules — lambda, pBR322, T7 and pUC19 — with the new enzyme and commercial Ava II (Fig. 3).

Sequencing. The precise cleavage structure was established by running a Maxam–Gilbert sequence gel [9] from the *Pst* I and *Hind* III fragment of pKL300 plasmid — which was derived from pKO-1 plasmid [10] — known to contain *Ava* II site labelled with ($\alpha^{32}\text{P}$) dATP by Klenow polymerase. The same end-labelled DNA fragment was then digested with the new enzyme and electrophoresed along-side the sequence ladder. This experiment unambiguously revealed that the cleavage occurred after the first Guanine nucleotide of the palindromic GG/A/T/CC pentanucleotide recognition sequence generating 5' protruding ends (Fig. 4).

Optimal conditions for enzyme activity. The enzyme activity was highlighted in low, medium and high ionic strength buffers [6] demonstrated that the enzyme activity is equally good in low and medium ionic strength buffer. The activity tests gave the best results at 37 °C and on pH 7.8. Heat inactivation was observed above 55 °C (data not shown).

Discussion

As a successful result followed by the finding of SmuC I a new type two restriction endonuclease was isolated and partially described. The accurate purification procedure was out of our interest, because of the large number of its isoschizomer but taking advantage of the method to eliminate non-specific nucleases from the enzyme preparation [8] — developed in the authors' laboratory — allowed us to determine the basic properties of the enzyme. The yield of the enzyme was about 600 units/gramm of wet cells. The recognition sequence is GG/A/T/CC, the enzyme cleaves before the second Guanine nucleotide within the recognition sequence in the 5' to 3' direction on both strand generating 5' extension cohesive termini.

In addition to the practical usefulness, a theoretical implication has to be mentioned as well. After *SmuC* I, *SmuE* I is the second restriction endonuclease which was found in a *Streptococcus mutans* strain with the similarity to *Anabaena variabilis* (*SmuE* I isoschizomer of *Ava* II, *SmuC* I isoschizomer

of *Ava III*). The possibility that these two evolutionary so different organism — a blue-green bacterium and a *Streptococcus* species — possess probably the same restriction-modification system may give rise to numerous other questions yet to be answered. Our work is in progress to descry the third enzyme — the isoschisomer of *Ava I* — in *Streptococcus mutans*, which would mean a perfect accomplishment to this interesting analogy.

REFERENCES

1. Westergreen, G., Emilson, C. G.: Arch Oral Biol **22**, 533 (1977).
2. Davidson, J. R., Blewins, W. T., Feary, T. W.: Antimicrob Agents Chemother **9**, 145 (1976).
3. Murray, K., Hughes, S. G., Brown, J. S., Bruce, S. A.: Biochem J **159**, 317 (1976).
4. Hughes, S. G., Bruce, T., Murray, K.: Biochem J **185**, 59 (1980).
5. Smith, H. O., Nathans, D.: J Mol Biol **81**, 419 (1973).
6. Sambrook, J., Fritsch, E. F., Maniatis, T.: In: Molecular Cloning — A Laboratory Manual I–II–III, Cold Spring Laboratory Press, Cold Spring Harbor 1989. pp. 5.3–5.32.
7. Helling, R. B., Goodman, H. M., Boyer, H. W.: J Virol **14**, 1235 (1974).
8. Geck, P., Molnár, A., Nász, I.: Acta Microbiol Hung **34**, 241 (1987).
9. Maxam, A. M., Gilbert, W.: In Grossman, L., Moldave, K. (eds): Methods in Enzymology. Vol 65. Academic Press, New York 1980. Part I, pp. 499–560.
10. McKenney, K., Shimatake, H., Court, P., Schmeissner, U., Rosenberg, M.: In Chirikjian, J., Papas, T. (eds): Gene Amplification and Analysis. Vol. 2. Elsevier, North-Holland, New York 1981. p. 383.

CLONAL DISTRIBUTION OF K1 AND K5 ANTIGEN POSSESSING *ESCHERICHIA COLI* ISOLATES

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(Received July 4, 1990)

A significant difference was observed in the occurrence of the examined markers (Col⁺, ColV⁺, Hly⁺, Aer⁺, Ab^R) and in the plasmid carrier state between strains with and without K1 and K5 antigens. Plasmids of the same size were harboured by serotypes possessing K1 and K5 antigens, e.g. among O1 : K1 : H⁺ strains plasmids of 60–79 Md, among O1 : K1 : H7, O18ac : K1 : H7, O45 : K1 : H7 and O83 : K1 : H⁺ strains plasmids of 80–95 Md were frequent. The average plasmid number was higher in K1 strains than in K5 strains. In serogroup O1 the frequency of the plasmid carrier state was associated with the O serogroup and not with the K antigen. The plasmid number in K5 of serogroups O6 and O18 was lower than in K5⁺ strains. Plasmids of 80–95 Md were predominant among the strains derived from blood and cerebrospinal fluid, whereas these plasmids were rare among the K1 and K5 strains isolated from other sources. Plasmids of 60–79 Md were frequent among strains derived from different sources. The 30–40 Md plasmids were relatively frequent among strains isolated from urine. In contrast with literary data, O1 : K1 : H⁺, O1 : K1 : H7 and other frequent serotypes consisted of different clones. Different clones were found within a single serotype, too.

The role of K1 and K5 antigens in pathogenicity was verified by literary data and also by our examinations [1–6]. Probably the linkage between K1 and K5 antigens and certain O antigens may result in the spread of some clones of increased pathogenicity. The purpose of the present study was to

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examine by complex typing the occurrence of certain clones or subclones. Determination of K1 and K5 antigens by specific phages allowed the Phage Laboratories of the Public Health Stations to select the strains for phage sensitivity examination.

Materials and methods

Bacterial strains. Complex typing of 439 *Escherichia coli* strains was performed; out of them 265 strains possessed antigen K1, 108 strains antigen K5 and 66 strains were K1-K5⁻. Complex typing of further 412 K1-K5⁻ strains was done without plasmid examination. The strains were isolated from specimens of hospitalized patients suffering from various diseases.

Serotyping. Determination of O and H antigens was carried out by the method of Ørskov and Ørskov [3].

Determination of antigens K1 and K5. Specific K1 phages and K5 phage were used. K1 phages A, B, C, D and E were propagated on strain *E. coli* U 9/41 [7], the K5 specific phage [8] was propagated on strain *E. coli* EH 342. Phages were employed undiluted and in RTD. Broth cultures of the strains tested were incubated at 37 °C for 2 h and poured on agar plate. After the culture had dried the phages were spotted on the plate, incubated at 37 °C for 5 h and the results were read. Lytic reactions showed K1 or K5 positivity.

Phage typing was carried out according to the method of Milch and Gyenes [9].

Colicin production and colicin typing was done by the method of Milch and Gyenes [9] using Col V⁺ and Col V⁻ strains (WS711nal, K12nal, 167-1, K12nal, 59-5, Tr41, Tr30-17) [10].

Aerobactin production (Aer) was determined by the qualitative biological method of Carbonetti and Williams [11] as modified by Rabsch and Reissbrodt [12].

Haemolysin production (Hly) was tested using the method of Cowan and Steel [13].

Antibiotic resistance (Ab^R) was determined by the use of "Resistest" disks ("Human" Institute for Serobacteriological Production and Research, Hungary) to streptomycin, neomycin, kanamycin, gentamicin, tetracycline, ampicillin, co-trimoxazole, chloramphenicol, nitrofurantoin and nalidixic acid.

Plasmid DNA was prepared by the method of Kado and Liu [14].

Agarose gel electrophoresis was carried out by electrophoresis in vertical agarose gels according to Meyers et al. [15], in 0.7% Sigma agarose gel, 35 mA, 125 V for 3 h. The preparations were stained with ethidium bromide (1 mg/1000 ml) for 30 min, photographed by UV light.

Standard plasmids for molecular size estimation: *E. coli* V517 (1.4, 1.8, 2.0, 2.6, 3.4, 3.7, 4.8, 35.8 Md), [16], R27 112 Md, R1 62 Md, pSpl 140 Md.

For significance the chi-square test [17] was used. The difference was considered as significant if $P \leq 0.05$.

Results

The 265 strains possessing K1 antigen belonged to 26 different serotypes and 7 kinds of serotype undefined by O antigens (not typable or spontaneously agglutinating). The 108 K5 strains fell into 15 serotypes. The 478 K1-K5⁻ strains belonged to 29 serotypes and to serotypes containing untypable O antigen.

Figure 1 shows the occurrence of various markers in K1 and K5 strains. The K1 strains were colicinogenic in 78.8%, out of the colicinogenic strains 26.2% were Col V positive (21.5% of the total number of strains). Haemolysin was produced by 20% of the strains, aerobactin by 73.6% of the strains; resistance to one or more antibiotics occurred in 22.3%. The K5 strains were colicinogenic in 17.6% (out of these 57.9% were Col V positive (10.2% of the

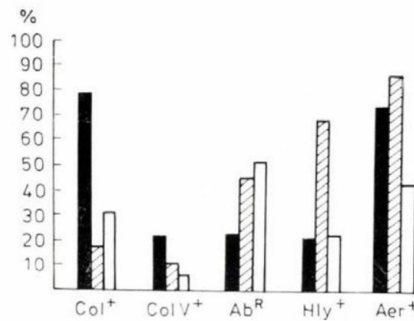


Fig. 1. Occurrence of Col⁺, Ab^R, Hly⁺ and Aer⁺ factors in *E. coli* K1, K5 and K1-K5- strains. No. of examined strains: K1 265; K5 108; K1-K5- 478; total 851. Solid columns, K1; shaded columns, K5; open columns, K1-K5-

total number of strains). Haemolysin production was recorded in 68.5%, aerobactin production in 86.1%, and antibiotic resistance in 45.4% of the cultures.

Colicinogenicity occurred more than fourfold higher among the K1 strains than among the K5 ones, and Col V⁺ strains were twice as frequent among the K1 strains. The differences were significant in both cases ($p < 0.001$ and $p < 0.01$, respectively). The occurrence of Col⁺ character among the K1-K5- strains was significantly lower than among the K1 ones ($p < 0.001$), but it was significantly more frequent than among the K5 strains ($p < 0.01$). Col V positivity was the rarest in the K1-K5- group, comparing to the K1+K5+ group ($p < 0.01$). Resistance to antibiotics was significantly more frequent in K5 than in K1 strains ($p < 0.01$). Antibiotic resistance was more frequent also in the K1-K5- group than among K1 strains ($p < 0.001$). The frequency of haemolysin production was significantly higher among K5 strains than among K1 ones ($p < 0.01$). The occurrence of Hly⁺ character was nearly the same

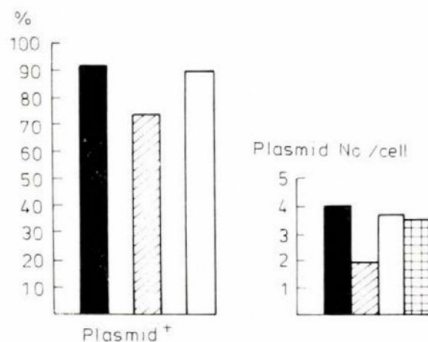


Fig. 2. Frequency of plasmid carrier state in *E. coli* K1, K5 and K1-K5- strains. No. of examined strains: K1 265; K5 108; K1-K5- 66; total 439. Solid columns, K1; shaded columns, K5; open columns, K1-K5-; hatched columns, total

among the K1-K5⁻ strains as among the K1 ones. Aerobactin production was found in a significantly lower percentage among K1 strains ($p < 0.02$) than among K5 ones. The Aer⁺ character in K1-K5⁻ strains was rarer than in the K1 strains ($p < 0.001$).

K1 strains had plasmids in 92.5%, the K5 strains in 73.1% (the difference was significant, $p < 0.001$). The plasmid carrier state in K5 strains was significant lower, ($p < 0.001$) than in the K1-K5⁻ strains (89.4%) (Fig. 2).

A total of 1073 plasmids of different molecular size was carried by the K1 strains, the number of plasmids per bacterial cell was 4.0. The number of plasmids of different molecular size carried by the K5 strains was 204, the plasmid number per cell was 1.9. The plasmid number per cell was 3.5 among K1-K5⁻ strains. The plasmid carrier state among the K5 strains was significantly rarer, than among the K1 and K1-K5⁻ strains ($p < 0.001$) (Fig. 2).

Analysing the markers according to serotypes (evaluating those serotypes only, which were represented at least by 10 strains) it was found (Tables I and II) that the Col⁺, Hly⁺ characters in O1:K1:H— strains corresponded to the average values in K1 antigen possessing strains, presented in Fig. 1, but the

Table I
Incidence of Col⁺ ColV⁺ Hly⁺ Aer⁺ Ab^R and plasmid number in E. coli K1 strains

Serotype	No. of examined strains	Col ⁺	ColV ⁺	Hly ⁺	Aer ⁺	Ab ^R	Plasmid/cell
O1 : K1 : H ⁻	36	31	4	14	2	5	5.8
O1 : K1 : H7	20	13	8	8	18	3	2.9
O2 : K1 : H5	14	9	9	—	14	11	0.6
O7 : K1 : H ⁻	16	9	1	13	16	1	6.4
O18ac : K1 : H7	14	14	5	—	13	3	1.4
O45 : K1 : H7	51	50	5	—	51	11	5.1
O83 : K1 : H ⁻	13	13	1	—	13	1	2.3
ONt : K1 : HNt	20	17	1	7	15	3	5.1

Table II
Incidence of Col⁺ ColV⁺ Hly⁺ Aer⁺ Ab^R and plasmid number in E. coli K5 strains

Serotype	No. of examined strains	Col ⁺	ColV ⁺	Hly ⁺	Aer ⁺	Ab ^R	Plasmid/cell
O6 : K5 : H ⁻	15	8	7	13	15	1	0.4
O6 : K5 : H1	17	—	—	10	14	14	2.3
O18ac : K5 : H ⁻	35	—	—	33	31	21	1.1
O75 : K5 : H ⁻	13	4	1	12	9	5	1.6

Aer⁺ character was much rarer (5.5%). The plasmid number per cell was higher than the average values (5.8). The Col⁺, Hly⁺ Aer⁺, Ab^R markers in O1 : K1 : H7 strains were of the average, whereas the plasmid number per cell (2.9) was lower than the average (4.0). Antibiotic resistance was more frequent among O2 : K1 : H5 strains (78.6%) than among other K1 serotypes. The plasmid/cell ratio was strikingly low: 0.6. The Hly character of the O7 : K1 : H— strains was frequent (81.2%) and the number of the carried plasmids was high (6.4). The O18ac : K1 : H7 strains were all colicinogenic, their plasmid/cell ratio being lower than the average: 1.4. Almost all O45 : : K1 : H7 and O83 : K1 : H— strains were Col⁺; Hly positivity was not detected, the plasmid/cell ratio was 5.1 among the O45 : K1 : H7 strains and 2.3 among the O83 : K1 : H— strains. The frequency of Col⁺ Hly⁺ Ab^R characters in not typable, ONt : K1 : H— strains was nearly the same as the average, the plasmid/cell ratio was 5.1 (Table I).

Among the K5 antigen possessing frequent serotypes the O6 : K5 : H— strains were Aer⁺ without exception; antibiotic resistance was rare (one strain), plasmid/cell ratio was 0.4. Col positivity was not found among the O6 : K5 : H1 strains, but antibiotic resistance was frequent (82.4%). The plasmid/cell ratio (2.3) was higher than that of the average in K5 strains. Col⁺ strains were not found among the O18 : K5 : H— strains, Hly positivity and antibiotic resistance was frequent. The plasmid/cell ratio was 1.1. Hly⁺ character was frequent also among the O75 : K5 : H— strains, the plasmid/cell ratio was 1.6 (Table II).

We examined whether plasmids of characteristic size were carried by the K1 and K5 strains. Table III shows the plasmid sizes occurred in the frequent serotypes. Out of the 36 O1 : K1 : H— strains plasmids of size 60–79 Md were carried by 20 strains. Out of the 20 O1 : K1 : H7 strains such plasmids were carried by 4 strains, the difference was significant ($p < 0.01$). The frequency of harbouring plasmids of 80–95 Md was higher among O1 : K1 : H7 strains than among O1 : K1 : H— strains ($p < 0.001$). Plasmids of this size were frequent among O2 : K1 : H5, O18ac : K1 : H7, O83 : K1 : H— and especially among O45 : K1 : H7 serotypes. The small plasmids were frequent among serotypes O1 : K1 : H—, O7 : K1 : H— and O45 : K1 : H7. The frequent plasmids of the K5 strains were the following: in O18ac : K5 : H— strains the 60–70 Md plasmids and in serotype O6 : K5 : H1 the small plasmids were frequent.

Plasmid-free strains were frequent among serotypes O2 : K1 : H5, O6 : K5 : H— and O18ac : K5 : H—, but they were rare among other serotypes. Out of the 33 K1 isolates plasmid-free strains were not found in 20 serotypes.

The average of plasmid number in the K1 strains was higher than in the K5 strains (Fig. 2). It was examined in different serogroups, whether this

Table III
Occurrence of plasmids in the frequent E. coli serotypes

Serotype	Plasmid size, Md								Plasmid free	No. of plasmids			No. of examined strains
	0.5-4.8	5.0-9.9	10-19	20-29	30-59	60-79	80-95	96		small	large	total	
O1 : K1 : H ⁻	106	50	3	5	17	20	3	3	1	159	48	207	36
O1 : K1 : H7	22	10	—	3	5	4	11	2	0	32	25	57	20
O2 : K1 : H5	1	—	—	—	—	—	6	1	6	1	7	8	14
O7 : K1 : H ⁻	72	11	—	—	15	4	1	—	0	83	20	103	16
O18ac : K1 : H7	7	2	—	2	2	1	5	1	3	9	11	20	14
O45 : K1 : H7	110	90	3	2	15	6	32	1	0	203	56	259	51
O83 : K1 : H ⁻	17	2	—	—	—	3	8	—	1	19	11	30	13
O6 : K5 : H ⁻	5	2	—	—	1	1	—	—	12	7	2	9	15
O6 : K5 : H1	18	11	—	—	—	1	5	4	0	29	10	39	17
O18ac : K5 : H ⁻	18	3	—	3	3	9	4	5	10	21	24	45	35

Table IV

Number of the carried plasmids in E. coli strains possessing the same O and different K antigens

O : K	Plasmid/cell	No. of examined strains
O1 : K1	3.8	70
O1 : K1 ⁻	5.3	3
O2 : K1	1.8	40
O2 : K5	6.0	3
O2 : K1 ⁻ K5 ⁻	2.6	8
O6 : K5	1.5	32
O6 : K5 ⁻	3.9	18
O18ac : K1	1.4	14
O18ac : K5	1.3	36
O18ac : K1 ⁻ K5 ⁻	1.7	6
O75 : K5	1.7	22
O75 : K5 ⁻	5.0	3

finding was really connected with the K antigen (Table IV). The average plasmid number was 3.8 in the O1 : K1 strains and 5.3 in the O1 : K⁻ strains; the average plasmid number was higher in the O1 serogroup both in the K1⁺ and K1⁻ strains. The frequency of plasmid carrier state is probably associated with the O antigen and the connection with K1 antigen is possibly illusory as the K1 antigen is linked to certain O antigens. The plasmid number was in O6 : K5 1.5, in O6 : K5⁻ 3.9 whereas in O75 : K5 1.7, in O75 : K5⁻ 5.0. The plasmid number in the K5 antigen-less strains was higher in these two serogroups. The plasmid numbers were 1.4, 1.3 and 1.7, respectively, in serogroups O18ac : K1⁺, O18ac : K5⁺ and O18ac : K1⁻ K5⁻; in this case the

Table V

Incidence of the frequent plasmids in E. coli K1 and K5 strains in percentage according to the source of isolation

Source	No. of examined strains	80-95 Md	60-70 Md	30-40 Md
Blood	7	71.4	—	14.3
Cerebrospinal fluid	2	100.0	—	—
Wound, pus	57	38.6	19.3	3.5
Organs	36	27.8	11.1	5.5
Urine	19	5.2	10.5	21.1
Nose, throat	91	34.1	24.2	6.6
Other	161	14.2	24.9	11.1
Total	373	28.7	21.4	8.8

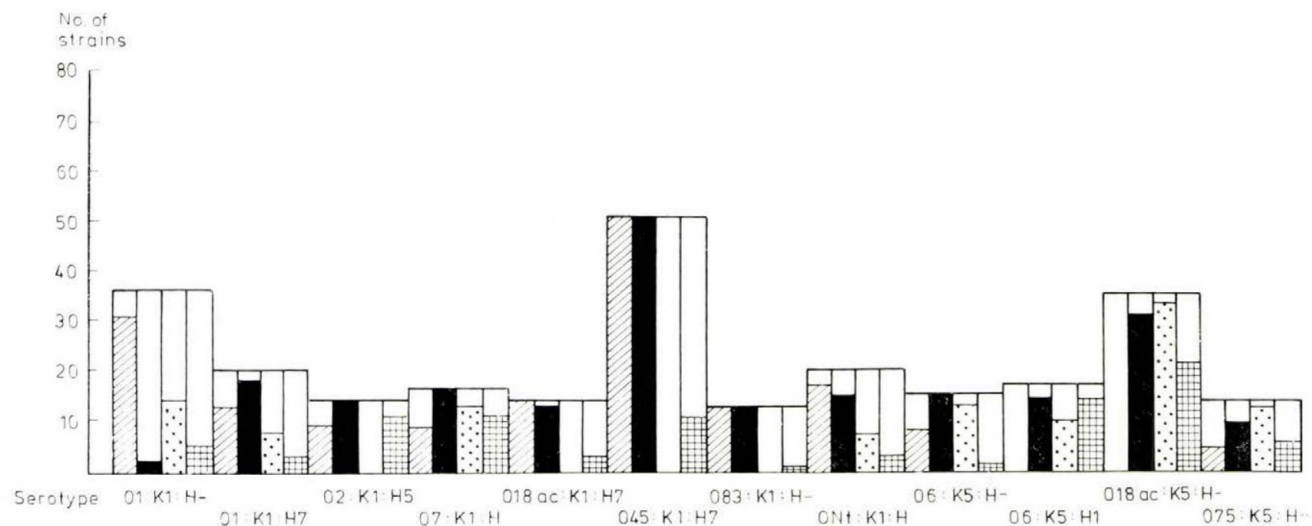


Fig. 3. Col, Aer, Hly, Ab^R markers in frequent *E. coli* serotypes. Shaded columns, Col⁺; solid columns, Aer⁺; spotted columns, Hly⁺; hatched columns, Ab^R; open columns, these markers absent

plasmid number of the K5⁺ strains was the smallest, though it was low in the K1⁺ and K1-K5⁻ strains, too. There was a difference between the O2 and the above mentioned serogroups concerning the plasmid/cell rate: K1 1.8, K5 4.5, K1-K5⁻ 2.6. The plasmid number was high in the O2 : K5 strains, in contrast with the other K5 antigen containing serogroups.

The frequency of plasmids of different sizes was examined according to the origin of the serotypes (Table V). Out of 7 strains isolated from blood, belonging to different serotypes, 80-95 Md plasmids were carried by 5. Two strains isolated from cerebrospinal fluid, of serotype O45 : K1 : H7 and O83 : K1H—, had a plasmid of 95 Md. Out of the 57 strains isolated from wound and pus (43 strains containing K1 antigen belonging to 17 serotypes and 14 strains containing K5 antigen, belonging to 5 serotypes) 80-95 Md plasmids were carried by 22 strains. Thirty-six strains isolated from different organs (stomach, uterus, prostate, cervix, trachea) were examined (27 K1 strains belonged to 13 serotypes, 9 K5 strains belonged to 5 serotypes); 80-95 Md plasmids were carried by 10 strains. Out of the 19 strains isolated from urine (14 K1 strains belonged to 9 serotypes, 5 K5 strains belonged to 4 serotypes) only one had a plasmid of 80-95 Md.

Plasmids of 60-79 Md were carried by 8 K1 and 3 K5 strains isolated from wounds and pus, 4 K1 strains derived from organs and 2 from urine.

It was examined by complex typing, whether a single serotype consisted of one or more specially pathogenic clones. The strains were considered as representing one and the same clone if they belonged to the same serotype and their phage patterns were identical (or they differed not more than in sensitivity to 2 phages) their colicin and aerobactin production was the same, their plasmid profiles were identical (or not more than 2 plasmids differed). In certain cases colicin and aerobactin production by strains of the same clones may differ, because these characters are controlled by plasmids.

The uniformity or diversity of the characters of the frequent serotypes derived from different areas of the country was examined by complex typing. The 11 most frequent serotypes according to their colicin, aerobactin, haemolysin production and antibiotic resistance are demonstrated in Fig. 3. All strains of serotypes O18ac : K1 : H7 and O83 : K1 : H— were colicinogenic; the majority of isolates in serotypes O1 : K1 : H—, O1 : K1 : H7, O2 : K1 : H5, O7 : : K1 : H—, O45 : K1 : H7, O6 : K5 : H— and O75 : K5 : H— produced colicin, while members of serotypes O6 : K5 : H1 and O18ac : K5 : H— were non-colicinogenic. Every frequent serotype produced aerobactin, except serotype O1 : K1 : H—; aerobactin negative strains were found in serotypes O1 : K1 : : H—, O1 : K1 : H7, O6 : K5 : H1, O18ac : K5 : H— and O75 : K5 : H—. The majority of strains were haemolysin producers, except those belonging to serotypes O18ac : K1 : H7, O45 : K1 : H7 and O83 : K1 : H—. Most of the strains of the examined serotypes were sensitive to antibiotics; antibiotic

Table VI
Phage types and plasmid profiles of the frequent E. coli K1 and K5 serotypes

Serotype	No. of phage types	Frequent phage types	Plasmid profile	Frequent plasmid (Md)
O1 : K1 : H ⁻	6	23; 15 23	one frequent	60-65
O1 : K1 : H7	10	4ab 13 23 26	heterogeneous	90-95
O2 : K1 : H5	4	12	of three kinds	90-95
O7 : K1 : H ⁻	2	4ab 13; 4ab 13 15	one frequent	35.8
O18ac : K1 : H7	9	13 23 25	one frequent	95
O45 : K1 : H7	4	3 13 23	one frequent	80-95
O83 : K1 : H ⁻	3	23 27	of two kinds	95
O6 : K5 : H ⁻	1	4ab 17	of two kinds	Plasmid free and 5.5
O6 : K5 : H1	2	4ab 13 16 17	one frequent	~7
O18ac : K5 : H ⁻	4	4ab 16 17 18	one frequent several different	65
O75 : K5 : H ⁻	3	4ab 16 17	heterogeneous	heterogeneous

resistance was frequent in serotypes O2 : K1 : H5, O6 : K5 : H1, O18ac : K5 : H⁻.

Table VI presents the results of the phage typing and plasmid profile analysis. Phage patterns varied from 1-10 in the different serotypes. The most heterogeneous phage patterns were found in serotypes O1 : K1 : H7 and O18ac : K1 : H7. The frequent plasmids varied between 5.5 and 150 Md and the 90-95 Md plasmid were carried frequently by the strains belonging to serotypes O1 : K1 : H7, O2 : K1 : H5, O18ac : K1 : H7, O45 : K1 : H7, O83 : K1 : H⁻.

On the basis of the above mentioned results it was probable that every serotype belonged to a different clone. It was assumed that one clone and its subclones were found in serotypes O1 : K1 : H⁻, O2 : K1 : H5, O7 : K1 : H⁻, O18ac : K1 : H7, O45 : K1 : H7, O83 : K1 : H⁻ and O6 : K5 : H⁻. In serotype O6 : K5 : H1 one frequent and two rare clones were found; several kinds of clones may have occurred in serotypes O1 : K1 : H7, O18ac : K5 : H⁻ and O75 : K5 : H⁻.

As an example we present the analysis of serotype O45 : K1 : H7 which occurred the most frequently. The strains belonged to 4 kinds of phage pattern, they produced colicin (with one exception) and all of them were Aer⁺ and Hly⁻. They were Ab^S (with 4 exceptions) and one plasmid pattern was frequent (95, 8.8, 5.5, 4.8, 2.8 Md). These organisms were spread in counties Fejér, Somogy, Nógrád and in Budapest. Strains possessing another, but nearly identical plasmid profile were also found, probably they were the subclones of the frequent clone (Table VII).

Table VII
Phage pattern and plasmid profile of E. coli O45 : K1 : H7 strains

Phage pattern	Plasmid profile, Md							No. of strains	Origin
	~100	70-95	40-65	38-40	6-29	3-5.5	1-2.8		
23		•			•	•	•	24	County Fejér, Budapest, Kaposvár, Salgótarján, Balassagyarmat, Nagyatád
13		•	•		•	•	•	10	Kaposvár, County Fejér, Marcali
3 23					•	•	•	4	Kaposvár, County Fejér
13 23				•	•	•	•	2	County Fejér
3 13 23		•	•	•	•	•	•	1	Balassagyarmat
4ab 13 23	•		•		•	•	•	1	County Fejér
4ab 13		•		•	•	•	•	1	County Fejér
12 13			•		•	•	•	2	County Fejér
4ab 23					•		•	1	County Fejér
3 4ab (7) 13 23						•	•	2	County Fejér, Marcali
2 3 7 8 13 23		•				•		2	Kaposvár
Nt		•				•	•	1	Kaposvár
Total								51*	

* All strains were Col⁺ Aer⁺ Hly⁻, 12 strains were Ab^R

Discussion

The clonal groups of K1 strains were studied by Achtman [18] by determining serotype, biotype, colicin and haemolysin production, OMP, LPS pattern, antibiotic resistance — and in certain cases — plasmid content. It was established that serotypes O1 : K1, O2 : K1 and O18 : K1 formed a group of subclones connected with each other, by having identical OMP pattern, though they belonged to different O groups. Our examinations did not agree with this finding; namely, the different serotypes belonged to different clones and different clones were probably present within the same serotype. Nimnich and Zingler [19] examining the phage pattern and biotype of *E. coli* O1 : K1 strains, found 2 kinds of clones: serotypes O1 : K1 : H7 : F11 and O1 : K1 : H— : F11. These results confirmed our observation that the O1 : K1 : H— and O1 : K1 : H7 strains were strictly different in respect to phage pattern, aerobactin production plasmid profile and in aerobactin production.

There were no data available in the literature on the inhibiting effect of plasmid acquisition by K5 antigen. The connection between plasmid number and K1 or K5 antigen is possibly due to the linkage between these antigens and certain O antigens and the plasmid acquisition is in connection with the O antigen. Mercer et al. [20] found that O18 : K1 and O1 : K1 strains showed similarity regarding their colicin production and OMP pattern and plasmids of

76–96 Md were frequently demonstrated. The 80–95 Md plasmids were frequent among O1 : K1 : H7 and O18 : K1 : H7 strains, but they were different according to phage pattern and other markers. It was found that the K1+ *E. coli* strains, derived from different areas, isolated from blood, cerebrospinal fluid and pus, carried 80–95 Md plasmids more frequently than the strains isolated from other sources.

In our previous work [21], in contrast with our present results, aerobactin production by the K1 strains was more frequent, than by the K5 strains; probably therefore, because in our present work serotype O1 : K1 : H— strains — which produced aerobactin rarely — were more frequent.

Valvano and Crosa [22] examined the presence of Col V plasmid and aerobactin system in an *E. coli* K1 strain causing meningitis of newborn babies. Aerobactin was produced by the examined strain, but plasmid determining aerobactin genes were not carried. It was demonstrated by hybridization that the aerobactin genes were located on a Hind III restriction fragment of 10.5 Kbp on the chromosome, and they showed conservation in a great extent with the homologous region of pCol V-K30. In our examinations with K1 containing strains of different serogroups, 4 Col V producer plasmid free strains were found. All strains produced aerobactin, too. Plasmids of 50–140 Md were carried by 32 colicin V producer strains and among them 90–95 Md plasmids were harboured by 23 strains. Hybridization experiments will demonstrate the presence of Col V and aerobactin genes on the chromosome in the 4 plasmid-free colicin V producing strains or in 6 strains carrying plasmid smaller than 25 Md.

Ørskov and Ørskov [23] reported serotypes frequent in sepsis, meningitis of newborns and in other infections. We also found these serotypes frequently, in addition serotype O45 : K1 : H7, which caused meningitis in newborn infants. Further in vitro and in vivo experiments are needed to clear up the pathogenic role of the 95 Md plasmid carried by *E. coli* K1 strains isolated from blood and cerebrospinal fluid. We obtained further data on the characters of pathogenic *E. coli* strains and the results of complex typing might determine the need and direction of epidemiological tasks by a further division within serotype.

REFERENCES

1. Robbins, J. B., McCracken, G. H., Gotschlich, E. C., Ørskov, F., Ørskov, I., Hanson, L. A.: *N Engl J Med* **290**, 1216 (1974).
2. Sarff, L. D., McCracken, G. H. Jr., Schiffer, M. S., Glode, M. P., Robbins, J. B., Ørskov, I., Ørskov, F.: *Lancet* **I**, 1099 (1975).
3. Ørskov, F., Ørskov, L.: *Acta Pathol Microbiol Scand* **83**, 595 (1975).
4. Cheasty, T., Gross, R. J., Rowe, B.: *J Clin Pathol* **30**, 945 (1977).
5. Evans, D. J. Jr., Evans, D. G., Höhne, C., Noble, M. A., Haldane, E. V., Lior, H., Joung, L. S.: *J Clin Microbiol* **13**, 171 (1981).
6. Cziráki, É., Milch, H., Vincze, J.: *Acta Microbiol Hung* **34**, 25 (1987).
7. Gross, R., Cheasty, U., Rowe, B.: *J Clin Microbiol* **6**, 548 (1977).

8. Gupta, D. S., Jann, B., Schmidt, G., Golecki, J. R., Ørskov, I., Ørskov, F., Jann, K.: *FEMS Microbiol Lett* **14**, 75 (1982).
9. Milch, H., Gyenes, M.: *Acta Microbiol Acad Sci Hung* **19**, 213 (1972).
10. Lafont, J. P., Dho, M., D'Hauterville, H. M., Brec, A., Sansonetti, Ph. J.: *Infect Immun* **55**, 193 (1987).
11. Carbonetti, N. H., Williams, P. H.: *Infect Immun* **46**, 7 (1984).
12. Rabsch, W., Reissbrodt, R. J.: *J Basic Microbiol* **25**, 663 (1985).
13. Cowan, S. I. (ed.): *Cowan and Steel's Manual for the Identification of Medical Bacteria*. 2nd Ed. Cambridge University Press, Cambridge 1974. p. 143.
14. Kado, C. L., Liu, S. T.: *J Bacteriol* **145**, 1365 (1981).
15. Meyers, J. A., Sanchez, D., Elwell, L. P., Falkow, S.: *J Bacteriol* **127**, 1529 (1976).
16. Macrina, F. L., Kopecko, D. J., Jones, L. R., Ayers, D. J., McCoven, M.: *Plasmid* **1**, 417 (1980).
17. Horváth, I.: *Quantitativ Microbiological Methods*. Akadémiai Kiadó, Budapest 1974.
18. Achtman, M.: In Korhonen, I. K., Dawes, E. A., Makela, P. H. (eds): *Enterobacterial Surface Antigens: Methods for Molecular Characterization*. Elsevier, Amsterdam 1985. p. 65.
19. Nimmich, W., Zingler, G.: *Med Microbiol Immunol* **173**, 75 (1984).
20. Mercer, A. A., Morelli, G., Henzenroeder, M., Kamke, M., Achtman, M.: *Infect Immun* **46**, 649 (1984).
21. Gadó, I., Milch, H., Czirók, É., Herpay, M.: *Acta Microbiol Hung* **36**, 51 (1989).
22. Valvano, M. A., Crosa, J. H.: *Infect Immun* **46**, 159 (1984).
23. Ørskov, F., Ørskov, I.: In Bergan, T. (ed.): *Methods in Microbiology*. Vol. 14. Academic Press, London 1984. p. 43.

POLYMORPHONUCLEAR CELL FUNCTION IMPAIRMENT IN PATIENTS WITH *CHLAMYDIA* *TRACHOMATIS* UROGENITAL INFECTIONS*

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(Received August 18, 1990)

The phagocytic and killing functions of polymorphonuclear cells (PMNs) and monocytes in patients with culturally and serologically proven *Chlamydia trachomatis* urogenital infections, and the distribution of peripheral blood T cell subsets in the same patients were analysed. A significant impairment of PMNs function was observed in infected patients as compared to the control group, whereas the monocyte function was not affected. In addition, a decrease of CD4⁺ cells frequency was observed in these patients. Since the PMNs are the most prominent cells observed at the site of *C. trachomatis* infection, we suggest that a defect of PMNs function may play a role in the host susceptibility to *C. trachomatis*.

Interest in *Chlamydia trachomatis* has increased in recent years because this agent is considered as one of the most common sexually transmitted pathogens [1]. Concerning the relationship of *C. trachomatis* with host immune system, polymorphonuclear leukocytes (PMNs) are the predominant inflammatory cells found at sites of infection and play a role in host defense against this microorganism [2].

In addition, some T cell activities are affected in patients with chlamydial infections. In this regard an impaired T suppressor function and B lymphocyte hyperreactivity were detected during chlamydial diseases [3, 4].

The purpose of this study was to evaluate the phagocytic and killing properties mediated by PMNs and monocytes isolated from patients with culturally and serologically proven *C. trachomatis* urogenital infections. At the same time the distribution of peripheral blood T cell subsets from same patients was analysed.

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* This work was presented in part at the 8th Meeting of International Society for Sexually Transmitted Diseases Research held in Copenhagen, Denmark, on September 10–13, 1989. The work was partly (60%) supported by a grant of Ministero della Ricerca Scientifica.

Materials and methods

Study population. Twenty-two patients (18 men and 4 women, mean age 30 years) attending the Institute of Dermatology, University of Bari were examined. Genitourinary infections due to *C. trachomatis* were assessed by culture and serology. Fourteen healthy volunteers (10 men and 4 women) without history of chlamydial infections were considered as the control group. None of the patients had history of recent antibiotic use.

Diagnostic procedures. Urethral specimens and endocervical specimens were collected into transport medium and inoculated into cycloheximide-treated monolayers of McCoy cells as previously described [5, 6]. After 2 to 3 days incubation at 37 °C chlamydial cytoplasmic inclusions were identified by the Lugol's iodine stain. Serodiagnosis of *C. trachomatis* infection was performed by the indirect fluorescent antibody technique (Chlamydia-Spot IF-kit, bio-Mérieux, France). Antibody titres $\geq 1:16$ for men and $\geq 1:64$ for women, determined on a single serum sample, were recorded as positive.

Phagocytosis by monocytes and PMNs. Peripheral blood mononuclear leukocytes from freshly drawn heparinized blood were obtained by density gradient centrifugation on Ficoll-Hypaque [7]. Monocytes were isolated by adherence to plastic Petri dishes. Polymorphonuclear leukocytes (PMNs) were harvested from human blood by 6% dextran sedimentation. Viabilities of the isolated cells exceeded 95%, as determined by exclusion of 1% trypan blue dye.

Candida albicans was used as test organism. The strain was cultured in Trypticase Soy Broth for 24 h, centrifuged at 3000 rpm for 10 min, washed three times with sterile phosphate buffered saline (PBS) and adjusted to a proper concentration. For phagocytosis assay *C. albicans* suspensions were mixed with monocytes or PMNs at the ratio 4:1 in the presence of 1.5% of autologous patient serum. The mixture was then incubated for 2 h in an end-over-end rotation at 37 °C. After 30 min incubation, aliquots of suspensions were washed clean of extracellular *C. albicans* and deposited on a glass slide by a cytocentrifuge. The slides were dried and stained with esterase or May-Grünwald-Giemsa. The smears were examined under oil immersion using a light microscope at 1000 $\times$ magnification; at least 200 cells/slide were counted for visual assessment of phagocytosis (number of phagocytes that have engulfed *Candida*).

Killing by monocytes and PMNs. Killing activity was evaluated according to the method described elsewhere [8]. Mixtures containing *C. albicans* and effector cells in a 4:1 ratio were incubated for 2 h at 37 °C. At various intervals samples of suspensions were taken out for the enumeration of viable *Candida*. After 2 h incubation, the cells were resuspended and colony count was performed to determine the total amount of *Candida* remaining. The tubes were centrifuged and colony count was determined in the supernatant. The colony counts were assessed by making 10-fold serial dilutions of the mixture in ice cold sterile distilled water by using the vortex in order to lyse the effector cells and to disperse microorganisms. Aliquots of 0.1 ml from each dilutions were spread in triplicate onto Sabouraud agar. After incubation at 37 °C for 48 h, the number of colony-forming units (c.f.u.) was determined. The percentage of *C. albicans* killed by effector cells was calculated as follows: c.f.u. at zero time — c.f.u. in 15 min supernatant = number of viable *Candida* in pellet in contact with monocytes or PMNs (X). 2 h total c.f.u. — c.f.u. in 2 h supernatant = number of viable cell-associated *Candida* at 2 h (Y). $X - Y \times 100 = \%$ *Candida* killed.

PBL surface marker analysis. T-cells subsets (OKT3+, OKT4+, OKT8+) in monocyte depleted mononuclear cells, were analysed by using fluorescein conjugated monoclonal antibodies (Ortho Diagnostic System, Milan, Italy) according to the manufacturer's instructions. Cytologic preparations were subsequently analysed under an Olympus BH-2 microscope equipped for epi-illumination fluorescence at 400 $\times$ magnification. The percentage of CD3+, CD4+ and CD8+ reacting cells was calculated out of the 300 cells for slide.

Statistics. Data were expressed as mean values $\pm$ standard deviation. The significance of differences between means was determined with Student's *t* test.

Results

As shown in Fig. 1, monocytes from patients were as effective at phagocytosing and killing *C. albicans*, as those from control group (phagocytosis $71.36\% \pm 5.66$ and $74.07\% \pm 4.8$, respectively, $p > 0.05$; killing $81.64\% \pm 6.73$ and $86.43\% \pm 4.22$, respectively, $p > 0.05$).

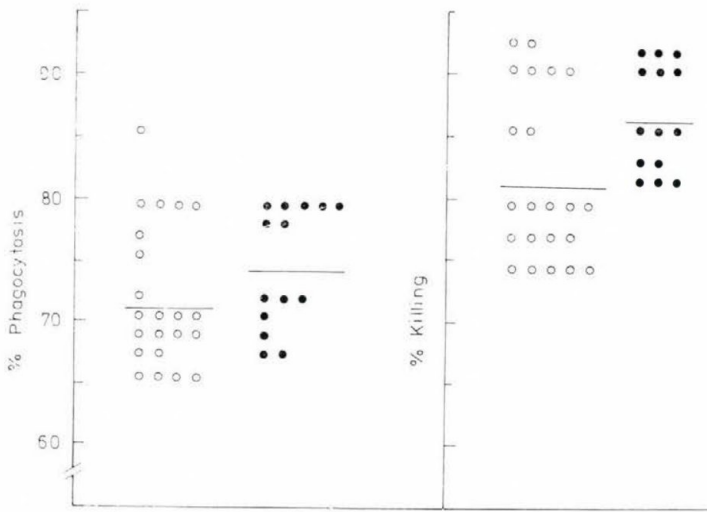


Fig. 1. Phagocytosis and killing activities of monocytes from patients with *C. trachomatis* urogenital infections (○) and from healthy persons (●). The horizontal bars indicate mean values

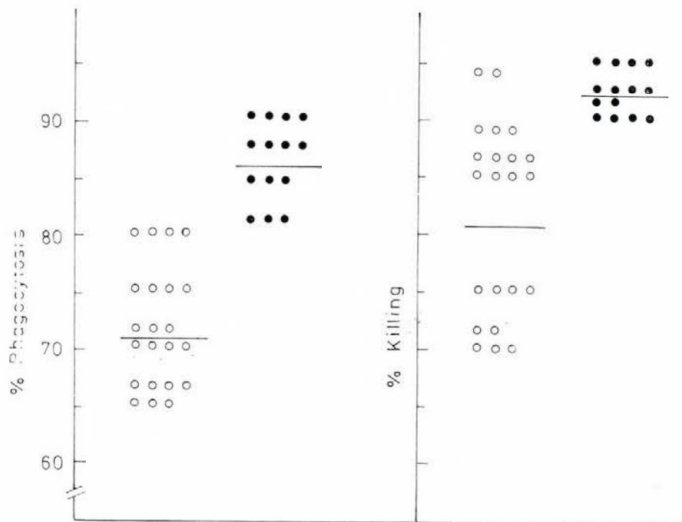


Fig. 2. Phagocytosis and killing activities of polymorphonuclear cells from patients with *C. trachomatis* urogenital infections (○) and from healthy persons (●). The horizontal bars indicate mean values

Figure 2 shows that PMNs functions, as determined by phagocytosis and killing of *C. albicans*, were depressed in patients with chlamydial infection as compared to control group (phagocytosis $71.68\% \pm 5.45$ and $86.36\% \pm 2.89$, respectively; killing 81.59 ± 8 and $92.43\% \pm 1.99$, respectively, $p < 0.01$).

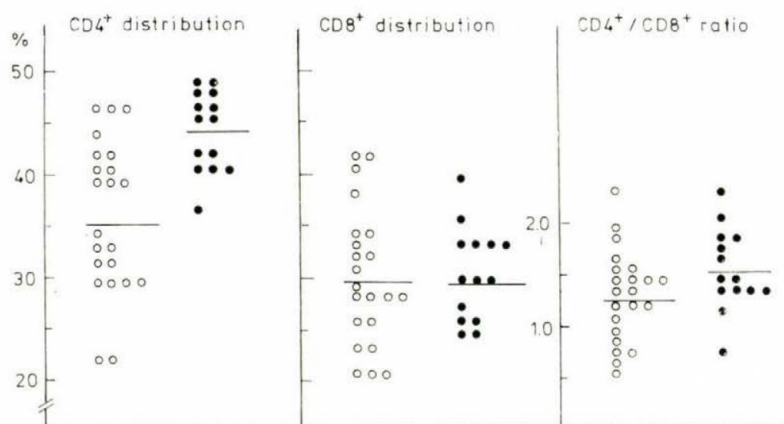


Fig. 3. CD4⁺, CD8⁺ distribution and CD4⁺/CD8⁺ ratio in patients with *C. trachomatis* urogenital infections (○) and in healthy persons (●). The horizontal bars indicate mean values

On the basis of previous reports suggesting that suppressor T cell activity could be impaired during chlamydial infection [3], the distribution of CD3⁺, CD4⁺ and CD8⁺ lymphocytes as well as the CD4⁺/CD8⁺ ratios in patients and controls were investigated. The difference between the mean values of T helper cells distribution within the two groups was statistically different ($35.5\% \pm 7.36$ and $44\% \pm 4.22$, respectively, $p < 0.01$), whereas the T suppressor cell percentage was similar in the two groups ($29.6\% \pm 6.46$ and $29.79\% \pm 5.19$, respectively, $p > 0.05$) (Fig. 3).

As far as the T helper/T suppressor ratio was concerned, differences between *C. trachomatis* infected individuals and healthy donors were not significant (1.27 ± 0.41 and 1.52 ± 0.38 , respectively).

Discussion

C. trachomatis is an emerging sexually transmitted pathogen in industrialized countries [5, 6, 9]. Several studies were conducted on the impact of *C. trachomatis* with the immune system and there were evidences that both humoral and cell-mediated immunity were involved in eliminating chlamydial infection. *C. trachomatis* induced an intense inflammatory response at sites of infection, and PMNs were the most prominent host effector cells observed whereas mononuclear phagocytes were not commonly seen [10, 11]. PMNs were then regarded to play a role in the localization of infection. In this respect, Bard and Levitt [12] demonstrated that 75% of PMNs bind *C. trachomatis* L2 serovar whereas Yong et al. [2] observed that intact PMNs were toxic to *C. trachomatis* biotypes B and L2. In view of the above concepts, in our study

the phagocytic and killing properties mediated by monocytes and PMNs isolated from peripheral blood from patients with urogenital infections due to *C. trachomatis* were analysed. Results indicated that in these patients the monocyte function, as assessed by phagocytosis and killing properties against *C. albicans* was not affected. On the other hand, a decrease of phagocytosis and killing activities by PMNs was observed in patients with *C. trachomatis* infections. Then, these findings lend further support to the role of PMNs in the host protection against *C. trachomatis*.

Noteworthy, studies on the T cell subset distribution revealed a significant decrease of CD4 + cells frequency when compared to that obtained from control group even if the CD4 +/CD8 + ratio was still within normal values. These results should be taken into consideration in view of the fact that T lymphocytes specifically react to *C. trachomatis* [13] and mediate optimum immunoglobulin production by stimulated B cells [14]. However, specific studies on immunoregulatory cells are required to assess T cell function in these patients. Taken together, this findings suggest that a defect of PMN function may play a role in the host susceptibility to *C. trachomatis* infection. Further studies will be required to confirm this hypothesis and to elucidate the mechanism by which *C. trachomatis* may escape host defenses.

REFERENCES

1. Schachter, J.: N Engl J Med **298**, 428 (1978).
2. Yong, E. C., Klebanoff, S. J., Kuo, C.: Infect Immun **37**, 422 (1982).
3. Andersen, P., Moller, B. R.: Scand J Infect Dis **4**, 19 (1982).
4. Levitt, D., Newcomb, R. W., Beem, M. O.: Clin Immunol Immunopathol **29**, 424 (1983).
5. Monno, R., De Vito, D., Vena, G. A., Mosca, A., Miragliotta, G.: Microbiologica **10**, 421 (1987).
6. Monno, R., Mosca, A., Vena, G. A., Foti, C.: In Proceedings of the European Society for Chlamydia Research, Esculapio Press, Bologna 1988, p. 51.
7. Boyum, A.: Scand J Clin Lab Invest **21**, (S97), 51 (1968).
8. Rosenwasser, L. J.: In Rose, N. R., Friedman, H., Fahey, J. L. (eds.): Manual of Clinical Laboratory Immunology. American Society for Microbiology, Washington D. C. 1986, p. 321.
9. Ladany, S., Sarov, I.: Eur J Epidemiol **1**, 235 (1985).
10. Lamont, H. C., Nichols, R. L.: In Nahmias, A. J., O'Reilly, J. (eds.): Immunology of Human Infection. Plenum Publishing Co., New York 1981, p. 441.
11. Kunitomo, D., Brunham, R. C.: Rev Infect Dis **7**, 665 (1985).
12. Bard, J., Levitt, D.: Clin Immunol Immunopathol **38**, 150 (1986).
13. Qvigstad, E., Digraanes, S., Thorsby, E.: Scand J Immunol **18**, 291 (1983).
14. Bard, J., Levitt, D.: Infect Immun **43**, 84 (1984).

PRINTED IN HUNGARY

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Acta Microbiologica Hungarica

VOLUME 38, NUMBER 2, 1991

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ACTA MICROBIOL. HUNG. AMHUEF 5 38 (2) 81-160 (1991) HU ISSN 0231-4622

ACTA MICROBIOLOGICA HUNGARICA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

Acta Microbiologica publishes reviews and original papers on microbiological subjects in English.

Acta Microbiologica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences

H-1117 Budapest, Prielle K. u. 19—35.

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Institute of Microbiology, Semmelweis University Medical School

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INDIRECT (PASSIVE) HAEMAGGLUTINATION TEST FOR ASSAY OF ANTIGEN AND ANTIBODY

(A REVIEW)

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Introduction

Antigen-antibody interactions constitute the specific methods for assay of antigen or antibody. The primary interaction of antigen with antibody is rarely visible. Visualization of the interaction is usually accomplished by labelling the antibody or the antigen with markers such as fluorescent dyes, radio-active, electron-dense or enzymatic markers [1]. The techniques used for measuring primary antigen-antibody interaction based on labelling of antibody or antigen are sensitive, specific, reproducible and have wide applications. Antigen-antibody reactions become visible when secondary reactions take place. For soluble antigens, the secondary reaction is visualized in the form of precipitation. Particulate antigens cause agglutination of the cells on which these antigens are present when secondary interaction takes place.

Agglutination tests are more sensitive than precipitation reactions [1]. Therefore, soluble antigens are coated onto larger insoluble particles such as polystyrene, latex, bentonite or red blood cells to increase the sensitivity of the test system for assay of antigen or antibody. Such reactions are referred as

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indirect (passive) agglutination. Inert particles such as polystyrene, latex or bentonite are difficult to coat, preserve and standardize and they have not displayed such wide range of usefulness that was initially expected of them [2]. On the other hand, erythrocytes have been found to be convenient carriers for antigen or antibody. Indirect agglutination with antigen coated erythrocytes is referred to as indirect (passive) haemagglutination (IHA) and erythrocytes coated with antibody are used in the reverse IHA test for assay of antigen.

The IHA test is versatile and it is possible to coat many antigens on the surface of erythrocytes. The IHA tests are simple, rapid, easy to perform, specific, sensitive and reproducible. A number of reactions and their application on the principle of reverse IHA have been reviewed by Coombs [3] and it has been described that such reactions hold great promise in the immunoassay technology. In these assays, the erythrocyte has been described as a label, tag or marker for antibody. The erythrocyte as a carrier replaces markers used for visualization of primary antigen-antibody reaction.

In recent years the IHA test for assay of antibody and reverse IHA test for assaying antigen have been widely used in bacteriology, virology, immunology, parasitology, mycology and vaccinology. For IHA test, certain antigens like polysaccharides may be directly coated on the erythrocytes just by exposing them to the antigen for a short time [4]. This type of coating is also possible with most proteins. The sensitivity of the test system can be improved by tanning of the erythrocytes [5]. The antigen can be attached to the surface of the cells through a covalent bond using certain chemicals like chromic chloride [6, 7], bis-diazotized benzidine [8, 9] and difluoro-dinitrobenzene [10]. The sensitivity and reliability of IHA test depends upon a number of factors such as sensitizing antigen, type of erythrocytes, the conditions of coating the erythrocytes with the antigen and performance of the test.

Sensitizing antigen

Polysaccharide antigens are absorbed directly onto normal erythrocytes. Capsular and somatic antigens from micro-organisms are also readily absorbed by erythrocytes [11]. However, some polysaccharides are efficiently absorbed after treating these with heat (100 °C for 1 h) or with dilute alkali or both. The nature of the alteration responsible for uptake of these antigens remains unknown [12]. The pneumococcal capsular polysaccharide type 6 is fixed to erythrocytes only after oxidized with periodate [13]. Baker et al. [14, 15] sensitized erythrocytes with purified type specific pneumococcal polysaccharide antigens using chromium chloride as a coupling agent. Lipopolysaccharides also needed prior treatment with alkali for sensitization of erythrocytes [16–18].

Protein antigens or immunoglobulins can be coated on erythrocytes with the help of tannic acid or be covalently linked to erythrocytes with some chemical agent [19]. The specificity of the IHA test depends upon the purity of the sensitizing antigen. The purified antigen should be used for sensitization of erythrocytes since contaminants would get absorbed on the erythrocytes and haemagglutination would occur with small amount of antibodies against the contaminants in the sera. Affinity-purified immunoglobulins are preferred for sensitization of erythrocytes with immunoglobulins to be used for reverse IHA test. In some instances globulins rather than antibodies have been found suitable for coating erythrocytes for reverse IHA test [20, 21].

Erythrocytes

Species of animals and collection of erythrocytes. Erythrocytes from mammalian or avian species are suitable for IHA test but sheep erythrocytes have been employed most extensively and successfully [11]. It is advantageous to use erythrocytes from the same species as the serum under test to avoid the complications produced by the presence in the serum of agglutinins directed against heterologous red blood cells [2]. If the erythrocytes are from the different species, the test sera are absorbed with the erythrocytes to remove the natural agglutinins [22–25].

The sensitivity and specificity of the IHA test depends upon the erythrocytes taken from different animal species. Sensitized human erythrocytes were found more sensitive than the sensitized sheep erythrocytes [26]. Certain workers found difficulties with rabbit, mouse and chicken erythrocytes [2, 27] while others found these cells satisfactory [28, 29]. Erythrocytes from pig, horse, ox, monkey and guinea pig have also been used successfully [2, 27–31]. It has been observed that the sensitivity of the IHA test varies if the erythrocytes are taken from different sheep [20, 22, 32, 33]. Hirata and Brandriss [34] reported similar results with rabbit erythrocytes.

Recently, avian erythrocytes which, being nucleated settle rapidly, have been found more suitable for IHA test. Turkey erythrocytes are now widely used in IHA test for the titration of tetanus antitoxin [35–37]. The results of IHA with sheep erythrocytes are obtained in 2 h while goose erythrocytes take 40 min only [20]. Parija et al. [38] got results of IHA test in 30–45 min using chick erythrocytes.

It should be checked that the sensitizing antigen does not cause direct haemagglutination of the erythrocytes. We could not standardize IHA or reverse IHA tests for the assay of pertussis antigens (pertussis toxin and filamentous haemagglutinin) or antibodies against these antigens since these two antigens caused haemagglutination of erythrocytes collected from sheep, rabbit, monkey, horse, goose, chicken, ox, guinea pig and mouse [39].

Usually one volume of blood is collected in 1.2 volumes of Alsever's solution [40]. The sheep blood can be stored at 4–8 °C in this solution for 3–4 weeks under aseptic conditions. Before use, the erythrocytes are washed at least 3 times with normal saline and the packed volume of cells is measured by centrifuging the cells at 2000 rpm for 7 min in a graduated centrifuge tube. The concentration of the cells has also been determined by measuring haemoglobin released from erythrocytes with distilled water [19, 41, 42].

Fixation of erythrocytes. Fixed erythrocytes are used in preference to unfixed erythrocytes in the IHA tests because of their resistance to lysis, stability on storage and ease of handling [22]. Unfixed or fresh cells can only be used for a limited period, say 3–4 weeks after which they become fragile, get contaminated or haemolysed. Moreover, the coupling agents damage unfixed cells and the resulting haemolysis interferes with subsequent titrations [43]. Furthermore, it has been found that the results obtained with fixed cells are generally more reliable and reproducible than those obtained with unfixed cells [44, 45]. The fixed cells may also be used in non-isotonic solutions, for example to detect viral antigens solubilized in detergents [31]. The fixed cells could be sensitized with the antigen at 56 °C increasing the sensitivity of IHA test as compared to unfixed cells which could be sensitized at 37 °C only [33]. The erythrocytes may be fixed before or after sensitization. However, it has been reported that fixed cells are not suitable for coupling the antibody with chromium chloride [31]. Ling et al. [46] could not fix erythrocytes even after coupling. However, Cranage et al. [31] were successful in fixing the antibody coupled erythrocytes with glutaraldehyde.

The erythrocytes may be fixed with several agents like formaldehyde [32, 47, 48], glutaraldehyde [22, 49], pyruvic aldehyde [50] and sulphosalicylic acid [51]. Of these, fixation with glutaraldehyde is rapid, easy to perform and reliable [22, 23, 33]. The glutaraldehyde fixation takes only 30 min as compared to 18–24 h period usually used for fixation with formaldehyde or pyruvic aldehyde. Glutaraldehyde fixed cells were found superior to formaldehyde fixed cells [49]. Moreover, glutaraldehyde fixed cells were sensitized with proteins without any coupling agent which was not possible for formaldehyde fixed erythrocytes [49, 52]. Parija and Ananthakrishnan [53] found erythrocytes treated sequentially with pyruvic aldehyde, tannic acid and glutaraldehyde more sensitive than those treated only with formaldehyde, glutaraldehyde or pyruvic aldehyde and subsequently tanned.

Preservation of erythrocytes. The unfixed cells are usually stored at 4–8 °C but the antibody linked red cells have been successfully cryopreserved by droplet freezing in liquid nitrogen [54]. The fixed and sensitized cells have been stored at 4–8 °C, –20 °C, –70 °C or have been lyophilized without loss of activity [2, 20, 55]. Glutaraldehyde fixed cells have been found superior to formaldehyde fixed cells for preservation also as the latter cells tended to

clump upon freezing and thawing making them unsuitable for the IHA test [49]. Glutaraldehyde fixed cells sensitized with Japanese encephalitis vaccine were stored at 4–8 °C for 2 years with approximately 4 times loss of sensitivity [55].

Diluents and treatment of test sera

The diluent for IHA test usually contains 0.5–1% normal rabbit serum (NRS). The sensitized cells are also suspended in this diluent. The use of 0.2% gelatin, bovine serum albumin, bovine gammaglobulin or rabbit gammaglobulin has also been reported [14, 19]. Shiori [56] proposed that the most probable mechanism of stabilization by NRS in the diluent is blocking of unoccupied sites that would otherwise tend to cause non-specific adherence. The addition of a protein stabilizer to the diluent is necessary to avoid non-specific agglutination and an increase in the concentration of the protein will often prevent the non-specific agglutination that occasionally persists at lower concentrations of the stabilizer [22]. The diluent containing NRS is superior to that containing polyvinyl-pyrrolidone [22] or Tween 80 and gum acacia [20].

The test sera are treated with an equal volume of washed and packed normal erythrocytes to remove natural agglutinins [22–24]. Though absorption of agglutinins from antisera has been found to be time consuming, tedious and loss in the sera, it could not be avoided even after replacement of sheep erythrocytes by human or chick erythrocytes for which most antisera have no naturally occurring agglutinins [22]. Ali-Khan [57] reported non-specific agglutination with human cells using tanned glutaraldehyde fixed cells sensitized with antigens from parasites. However, Maheshwari et al. [58] found it unnecessary to absorb the mouse sera with sheep erythrocytes.

The IHA test, like the agglutination reactions, is more efficient in assaying IgM antibodies [1]. Therefore, IHA titres are generally higher than the neutralization titres of the sera [22–24, 59–62] because the neutralization method measures IgG only [63]. The treatment of test sera with 2-mercaptoethanol, a disulphide bond reducing agent acting on IgM but not on IgG [64], has been done to obtain similar IHA and neutralization titres [22–25]. The use of 2-mercaptoethanol in the diluent has also been done to avoid the need for removing heterophile IgM agglutinins [2, 65, 66]. Maheshwari et al. [58] did not find any advantage of treating the mouse sera with 2-mercaptoethanol.

Sensitization of erythrocytes

The sensitization of erythrocytes with the antigen is the most important step in the preparation of erythrocytes for IHA tests. As mentioned already, the erythrocytes can be sensitized with polysaccharide antigens by simple

adsorption of antigen by the erythrocytes or the antigen may be coated on the erythrocytes by tanning. Further the antigen may be covalently linked/coupled to the erythrocytes with the help of certain chemical agents.

Direct adsorption of antigen by erythrocytes. Many polysaccharides like capsular uronic acid polymers, certain pneumococcal type specific polymers coat cells directly without any prior treatment while other like lipopolysaccharides of Gram-negative bacteria require prior treatment before adsorption [11]. The adsorption is simple incubation of the antigen with erythrocytes at 37 °C for 1 to 2 h in the presence of an isotonic level of electrolytes. The sensitivity of the adsorbed cells depended upon the concentration of antigen used and period of adsorption [67]. The erythrocytes usually take very small amount of antigen and after adsorption the cells are washed to remove excess antigen which would otherwise cause haemagglutination inhibition during titration. The surface of erythrocytes possess various receptors for different polysaccharides. When erythrocytes were exposed to 6 polysaccharide antigens of different specificity either in turn or simultaneously, the coated cells reacted with each of the panel of antisera [68]. The attachment of antigen to cells is not a simple charge phenomenon and fixation of polysaccharides is remarkably firm, the stability of coated cells is similar to that of the erythrocytes itself [11].

The IHA test by simple adsorption has been used for bacterial antigens of *Escherichia coli* [17, 18, 28, 69], *Pasteurella* sp. [29, 70], *Neisseria meningitidis* [71]; *Pseudomonas pseudomallei* [72]; of protozoal antigen from *Toxoplasma* [2], polysaccharide antigen of *Trichomonas vaginalis* [73]; viral antigens [74, 75] and antibiotics like penicillin [67]. Even certain proteins, haptens and nucleic acid (DNA) have also been coated on erythrocytes by direct adsorption [76–79].

Coating of antigens with tannic acid. Protein antigens may be coated on erythrocytes by simple adsorption but the sensitivity of IHA with such sensitized cells is very low [2]. The quantity of protein antigen being coated on erythrocytes increases after these are treated with tannic acid [56]. The major role of tannic acid is to increase the instability of the cells so causing a normally non-agglutinating reaction to agglutination [80]. The binding of antigen on tanned cells is somewhat reversible but it is found to be advantageous to employ this procedure rather than coupling proteins to red cells because the protein coated red cells are more stable than the protein coupled cells [19]. Usually more antigen is required for coupling than for coating.

Both types of unfixed erythrocytes may be used for sensitization with the help of tannic acid. A good reagent grade tannic acid is recommended for tanning [52]. A 1% stock solution of tannic acid may be used for not more than one month but it is preferable to make the solution freshly each time when the erythrocytes are to be sensitized [59, 81]. The tanned cells may be

stored for several days at 4–8 °C before coating [82, 83] while formalinized and tanned cells have been preserved for months without any loss [2].

After tanning the antigen is coated onto erythrocytes. The sensitivity of erythrocytes depends upon the conditions of sensitization of tanned erythrocytes with the antigen. It is preferable to determine the optimal conditions of sensitization of erythrocytes such as concentration of antigen, pH and period of sensitization [2, 19, 22, 33]. The sensitivity of the cells also depends upon the temperature of sensitization, the optimal temperature being 56 °C for fixed erythrocytes [33]. The sensitizations carried out at 56 °C, pH 7.2 for 1 h have been found suitable for various antigens like tetanus toxoid, diphtheria toxoid, antibodies against Japanese encephalitis virus and Japanese encephalitis vaccine [20, 33, 55, 58]. Stavitsky [19] found pH 6.4 suitable for most of the antigens when unfixed cells were used and pH 5.6 to 6.4 when formalinized cells were used. During sensitization, a very small amount of antigen gets coated onto erythrocytes [2]. Therefore, it has been suggested that the single antigen preparation may be used repeatedly for sensitization [19, 20], but it again varies with the antigens.

Coupling of antigen with chemical reagents. The antigen may be coupled to the red cells with a chemical agent resulting in cells having several advantages except that the cells are more fragile. The advantages include no desorption of antigen, the end point of IHA titrations are more sharp and the preparations can be used in the presence of complement [84]. The antigen can be coupled with the help of bisdiazobenzidine on fresh cells which are susceptible to haemolysis. Therefore the formalinized cells are preferred [2, 9]. The major step in this procedure is preparation of benzidine compound which is stored at –20 °C to –40 °C for use. The antigen is coupled by incubating the cells, bisdiazobenzidine and antigen together at room temperature for 15 min and then washing the sensitized cells [8, 85–87]. The preparations made with fresh cells can be stored at 3–4 °C for 4–5 h while the formalinized preparations may be stored for several weeks [84].

The other most widely used chemical agent for coupling of antigen to erythrocytes is chromium chloride. Only unfixed cells are used for coupling of antigen with chromium chloride. Scott et al. [88] have described the treatment of erythrocytes with trypsin before coupling to increase the sensitivity of the test. The erythrocytes coupled with antigen by chromium chloride can be stored at 4–8 °C for 15 days. Several applications of antibody coupled erythrocytes prepared in this way have been described [3]. Recently a rapid procedure for coupling protein antigens to red cells with chromium chloride has been described [89].

Another chemical agent which has been employed for coupling of antigen to erythrocytes is difluorodinitrobenzene [10, 90, 91].

Performance of the test

The sensitivity of the IHA test depends also upon the final concentration of sensitized erythrocytes. If the final concentration of sensitized erythrocytes is high, these show low sensitivity. The end points are more clear and easy to read. A final concentration of 0.5% sensitized erythrocytes has been found suitable as a compromise between sensitivity and readability [22, 33]. Several workers reported the use of 1% final concentration of sensitized cells [88, 92].

The IHA titrations can be performed in tubes, U or V type microtitration plates using equal volumes of the various dilutions of the test sample and sensitized cells. The reference preparation preferably in terms of International Units (IU) should be included in all the titrations to get the results in IU [22–25]. This would minimize the variations from test to test. Microtitration plates are now used most commonly and 2-fold dilutions of the samples are made in the wells of the plates. The controls include the addition of non-sensitized cells to the first dilution of the sample and diluent. The sensitized cells are also added to diluent alone. The haemagglutination reaction is graded as described [22, 33]. For a valid test there must be no agglutination of non-sensitized cells by the sample nor of sensitized or non-sensitized cells in diluent alone.

The antibody is assayed by IHA test using antigen sensitized erythrocytes. For assaying antigen the reverse IHA test employing antibody coated erythrocytes is used. The antigen can also be assayed by IHA inhibition test as described [19, 59, 93, 94]. For assaying toxoids, a particular dose of known toxoid causing IHA inhibition of fixed units of antitoxin has been employed for titration of unknown toxoids [59, 93, 94]. Alternatively, the other method described by Stavitsky [19] using several series of dilutions of antiserum may be employed.

REFERENCES

1. Zmijewski, C. M., Bellanti, J. A.: In Bellanti, J. A. (ed.): *Immunology — Basic Processes*. Saunders, Philadelphia 1985. pp. 16–0175.
2. Herbert, W. J.: In Weir, D. M. (ed.): *Handbook of Experimental Immunology*. Blackwell Scientific Publications, Oxford 1978. Parts 20.1–20.20.
3. Coombs, R. R. A.: In Voller, A., Bartlett, A., Bidwell, D. (eds.): *Immunoassays for the 80's*. MTP Press, Lancaster 1981. pp. 17–34.
4. Keogh, E. V., North, E. A., Warburton, M. F.: *Nature* **161**, 687 (1948).
5. Boyden, S. V.: *J Exp Med* **93**, 107 (1951).
6. Fudenberg, H. H., Drews, G., Nisonoff, A.: *J Exp Med* **119**, 151 (1964).
7. Gold, E. R., Fudenberg, H. H.: *J Immunol* **99**, 859 (1967).
8. Pressman, D., Campbell, D. H., Pauling, L.: *J Immunol* **44**, 101 (1942).
9. Lavergne, M., Mangalo, R., Raynaud, M.: *Ann Inst Pasteur* **109**, 94 (1965).
10. Ling, N. R.: *Immunology* **4**, 49 (1961).
11. Landy, M.: In William, C. A., Chase, M. W. (eds): *Methods in Immunology and Immunochemistry*. Vol IV. Academic Press, New York 1977. pp. 26–30.
12. Neter, E.: *Bacteriol Rev* **20**, 166 (1956).

13. Rebers, P. A., Hurwitz, E., Heidelberger, M., Estrada-Parra, S.: *J Bacteriol* **83**, 335 (1962).
14. Baker, P. J., Stashak, P. W., Prescott, B.: *Appl Microbiol* **17**, 422 (1969).
15. Baker, P. J., Stashak, P. W.: *J Immunol* **103**, 1342 (1969).
16. Neter, E., Westphal, O., Luderritz, O., Gorzynski, E. A., Eichenberger, E.: *J Immunol* **76**, 377 (1956).
17. McCabe, W. R., Greely, A.: *J Immunol* **108**, 601 (1972).
18. McCabe, W. R., Demaria, A., Berherich, H., Johns, M. A.: *J Infect Dis* **158**, 291 (1988).
19. Stavitsky, A. B.: In William, C. A., Chase, M. W. (eds): *Methods in Immunology and Immunochemistry*. Vol. IV. Academic Press, New York 1977. pp. 30-41.
20. Gupta, R. K., Mehta, V. K., Gupta, V. K., Misra, C. N., Saxena, S. N.: *J Biol Stand* **15**, 271 (1987).
21. Gupta, R. K., Mehta, V. K., Gupta, V. K., Misra, C. N., Saxena, S. N.: *J Biol Stand* **15**, 287 (1987).
22. Peel, M. M.: *J Biol Stand* **8**, 177 (1980).
23. Gupta, R. K., Maheshwari, S. C., Singh, H.: *J Biol Stand* **12**, 137 (1984).
24. Gupta, R. K., Maheshwari, S. C., Singh, H.: *J Biol Stand* **12**, 145 (1984).
25. Gupta, R. K., Maheshwari, S. C., Singh, H.: *J Biol Stand* **13**, 151 (1985).
26. Steele, A. S. V., Coombs, R. R. A.: *Int Arch Allergy* **25**, 11 (1964).
27. Felton, F. G., Scott, L. V.: *J Immunol* **86**, 42 (1961).
28. Neter, E., Bertram, L. F., Zak, D. A., Nurdock, M. R., Arbesman, C. E.: *J Exp Med* **96**, 1 (1952).
29. Wright, G. G., Feinberg, R. J.: *J Immunol* **68**, 65 (1952).
30. Keskar, M., Natu, M., Borkar, M. B.: *Indian J Med Res* **73**, 74 (1981).
31. Cranage, M. P., Gurner, B. W., Coombs, R. R. A.: *J Immunol Methods* **64**, 7 (1983).
32. Galazka, A., Abgarowicz, A.: *Epidemiol Rev* **21**, 237 (1967).
33. Gupta, R. K., Maheshwari, S. C., Singh, H.: *J Biol Stand* **12**, 11 (1984).
34. Hirata, A. A., Brandriss, M. W.: *J Immunol* **109**, 641 (1968).
35. Bistoni, F., Pitzurra, M., Marconi, P.: *Boll Ist Sieroter Milan* **62**, 575 (1983).
36. Marconi, P., Pitzurra, M., Bistoni, F.: *Boll Ist Sieroter Milan* **62**, 567 (1983).
37. Marconi, P., Pitzurra, M., Bistoni, F.: In Nistico, P., Mastroeni, P., Pitzurra, M. (eds): *Seventh International Conference on Tetanus*. Gangemi Publ. Co., Rome 1985. pp. 259-273.
38. Parija, S. C., Kasinathan, S., Rao, R. S.: *J Trop Med Hyg* **92**, 221 (1989).
39. Gupta, R. K.: Development of an Improved Pertussis Vaccine. Ph. D. Thesis. Shimla, Himachal Pradesh University 1987.
40. Alsever, J. B., Ainslie, R. B.: *New York State J Med* **41**, 126 (1941).
41. Kent, J. F., Bukantz, S. C., Rein, C. R.: *J Immunol* **53**, 37 (1946).
42. Battisto, J. R., Chase, M. W.: *J Exp Med* **118**, 1021 (1963).
43. Onkelinx, E., Meuldermans, W., Janiou, M., Lontie, R.: *Immunology* **16**, 35 (1969).
44. Fulthorpe, A. J.: *J Hyg* **55**, 382 (1957).
45. Kyselova, M., Libich, M., Srbova, H.: *Z Immun Allergy Klin Immunol* **139**, 228 (1970).
46. Ling, N. R., Bishop, S., Jefferis, R.: *J Immunol Methods* **15**, 279 (1977).
47. Csizmas, L.: *Proc Soc Exp Biol Med* **103**, 157 (1960).
48. Daniels, T. M., Weyand, J. G. M., Stavitsky, A. B.: *J Immunol* **90**, 741 (1963).
49. Bing, D. H., Weyand, J. G. M., Stavitsky, A. B.: *Proc Soc Exp Biol Med* **124**, 1166 (1967).
50. Ling, N. R.: *Br J Haematol* **7**, 299 (1961).
51. Brecht, H.: *J Immunol* **101**, 18 (1968).
52. Singh, H., Maheshwari, S. C., Gupta, R. K.: In Saxena, S. N. (ed.): *Proceedings of the National Seminar on Quality Control of Vaccines*. Arun Publishing House, Chandigarh 1985. pp. 113-131.
53. Parija, S. C., Ananthakrishnan, N.: *J Med Microbiol* **19**, 95 (1985).
54. Pegg, D. E., Diaper, M. P., Scholey, S. E. G., Coombs, R. R. A.: *J Immunol Methods* **54**, 331 (1982).
55. Gupta, R. K., Misra, C. N., Mehta, V. K., Gupta, V. K., Rao Bhau, L. N., Singh, G., Goyal, D., Saxena, S. N.: *Indian J Med Res* (1990); In Press.
56. Shiori, K.: *Jap J Exp Med* **34**, 345 (1964).
57. Ali-Khan, Z.: *Int J Parasitol* **4**, 549 (1974).
58. Maheshwari, S. C., Sharma, S. B., Ahuja, S., Saxena, S. N.: *J Biol Stand* **15**, 315 (1987).
59. Tasman, A., Ramshorst, J. D. van, Smith, L.: *Antonie van Leeuwenhoek* **26**, 413 (1960).
60. Surján, M., Nyerges, G.: *Z Immun Exp Ther* **124**, 390 (1962).
61. Levine, L., Wyman, L.: *Am J Hyg* **80**, 314 (1964).
62. Chatterjee, S. C.: *Indian J Med Res* **52**, 1241 (1964).
63. Ourth, D. D., MacDonald, A. B.: *Immunology* **33**, 807 (1977).

64. Deutsch, H. F., Morton, J. I.: *Science* **125**, 600 (1957).
65. Adeniyi-Jones, C.: *Lancet* **1**, 188 (1967).
66. Greenwood, B. M.: *Clin Exp Immunol* **6**, 197 (1970).
67. Ley, A. B., Harris, J. P., Brinkley, M., Liles, B., Jack, J. A., Cahan, A.: *Science* **127**, 1118 (1958).
68. Landy, M.: *Am J Publ Hlth* **44**, 1059 (1954).
69. Neter, E., Cohen, E., Westphal, O., Luderitz, O.: *J Immunol* **82**, 85 (1959).
70. McVey, D. S., Loan, R. W., Purdy, C. W., Richards, A. E.: *Am J Vet Res* **50**, 443 (1989).
71. Hammond, B. W., Kingsbury, D. T., Weiss, E.: *J Immunol* **101**, 808 (1968).
72. Ashdown, L. R., Johnson, R. W., Koehler, J. M., Cooney, C. A.: *J Infect Dis* **160**, 253 (1989).
73. Satapathy, G., Kar, S. K., Samantaray, J. C., Panda, S. K.: *Genitouri Med* **64**, 110 (1988).
74. Burnet, F. M.: *Br J Exp Pathol* **27**, 244 (1946).
75. Burnet, F. M., Anderson, S. G.: *Br J Exp Pathol* **27**, 236 (1946).
76. Sindo, T., Wakakura, K.: *Jap J Exp Med* **22**, 285 (1952).
77. Lawlis, J. F.: *Proc Soc Exp Biol Med* **98**, 300 (1958).
78. Levine, B. B., Levytska, V.: *J Immunol* **98**, 648 (1967).
79. Bullock, W. E., Kantor, F. S.: *J Immunol* **94**, 317 (1965).
80. Pirofsky, B., Cordova, M., Imel, T. L.: *J Immunol* **89**, 767 (1962).
81. Borduas, A. G., Grabar, P.: *Ann Inst Pasteur* **34**, 903 (1953).
82. Stavitsky, A. B.: *J Immunol* **72**, 360 (1954).
83. Linz, R., Lecocq, E., Mandelbaum, E.: *Ann Inst Pasteur* **101**, 367 (1961).
84. Arquilla, E. R.: In William, C. A., Chase, M. W. (eds): *Methods in Immunology and Immunochemistry*. Vol. IV. Academic Press, New York 1977. pp. 41-47.
85. Stavitsky, A. B., Arquilla, E. R.: *J Immunol* **74**, 306 (1955).
86. Gordon, J., Rose, B., Sehon, A. H.: *J Exp Med* **108**, 37 (1958).
87. Butler, W. T.: *J Immunol* **90**, 663 (1963).
88. Scott, M. L., Thornley, M. J., Coombs, R. R. A., Bradwell, A. R.: *Int Arch Allergy Appl Immunol* **64**, 202 (1981).
89. Savelkoul, H. F. J., Greeve, A. A. M., Rijkers, G. T., Marwitz, P. A., Benner, R.: *J Immunol Methods* **111**, 31 (1988).
90. Fulthorpe, A. J., Parke, J. A. C., Tovy, J. E., Monckton, J. C.: *Br Med J* **1**, 1049 (1963).
91. Johnson, H. M., Brenner, K., Hall, H. E.: *J Immunol* **97**, 791 (1966).
92. Hardegree, M. C., Barlie, M. F., Pittman, M., Maloney, C. J., Schofield, F., MacLennan, R.: *Bull WHO* **43**, 461 (1970).
93. Fulthorpe, A. J.: *Immunology* **1**, 365 (1958).
94. Gupta, R. K., Maheshwari, S. C., Singh, H.: *J Biol Stand* **13**, 355 (1985).

ISOLATION OF AN INFECTIOUS ONCOGENIC ADENOVIRUS STRAIN FROM THE STIMULATED LYMPHOCYTES OF A PATIENT WITH BLADDER TUMOUR

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(Received March 27, 1990)

In attempts to grow viruses from tumour cells and circulating lymphocytes, we isolated an oncogenic infectious adenovirus type 18 strain from the phytohaemagglutinin-stimulated peripheral T-lymphocytes of a patient with bladder tumour. The virus genome is assumed to play certain role in the development of the tumour in interaction with other DNA viruses and the damaged immune system.

It is well known that the tumour cells and circulating lymphocytes of patients with malignant urogenital tumours can carry virus components, latent viruses, but any attempts to gain infectious viruses in the above cases have been unsuccessful. In the present study we attempted to isolate infectious virus from the tumour cells of patients with urogenital tumours and from their circulating T-lymphocytes.

Patients and methods

Patients. We tried to grow viruses from the surgically removed tumours and separated lymphocytes of patients with various types of malignant urogenital tumours. Of the 25 patients 11 had kidney carcinoma, 10 bladder carcinoma, and 4 bladder papilloma. Additionally, lymphocytes of patients stimulated in vitro with phytohaemagglutinin (PHA) were examined. Of the 30 patients 13 had kidney carcinoma, 9 bladder carcinoma, 3 prostate carcinoma, and 5 prostate adenoma.

Separation and extraction of tumour cells. Pieces of tumourous tissue taken out surgically were washed several times in saline containing antibiotics, cut into tiny pieces with sterile scissors and suspended in saline. Then the tissue fragments were allowed to sediment and the supernatants containing cells or conglomerations of cells were transmitted to the cell-cultures. Larger tissue pieces were ground with quartz sand, centrifuged and the supernatants containing cell extract were used as inoculum.

Separation, extraction and stimulation of lymphocytes. Blood specimens taken with 0.5 mg/ml heparin without preservative were separated by filtration on fibreglass and sedimented at room temperature. The lymphocyte-rich supernatant containing nearly solely T lymphocytes and precursor cells was removed. One part of the lymphocytes was directly taken to the cell cultures, the other part was ground with quartz sand, centrifuged and supernatants were applied as inoculum in cell cultures. For stimulating the T lymphocytes Phyto-

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haemagglutinin (Difco P) in 1 : 3000 final dilution was added to the lymphocyte suspensions and cell cultures were incubated in Parker 199 solution at 37 °C for 3 days. One part of these stimulated cells was directly taken to the cell-cultures while the other part of them was ground and the cell extracts were applied as inoculum.

Cell-cultures. The cell-line marked HEP-2 originated from the USA and was kept in our own laboratory for years. The human amnion primer cell-culture was made by ourselves from fresh placenta amnion epithelium of mature newborns with the known trypsinizing method. The human embryo fibroblast primary culture was made in our laboratory from embryos obtained by surgical abortion.

Virus-isolation and identification. The separated tumour cells and tumour cell extracts, the patients' separated T-lymphocytes, their extracts, stimulated T-lymphocytes and their extracts were placed on susceptible primary and permanent cell cultures. To follow the virus effect, the cultures were studied under the light microscope every 1 or 2 days for 8 weeks. To sustain the cultures, the maintenance solution was changed every week. The result of virus isolation was considered negative, if no cytopathic effect had appeared after three or four blind passages. The grown virus was identified with complement-fixing reaction, agar gel precipitation, virus neutralization and restriction enzyme analysis.

Virus antisera. Hyperimmune viral antisera were prepared in rabbits against adenovirus and herpesvirus of different types (HSV-1, 2; adenovirus type 1, 5, 6, 7, 8, 10, 14; 12, 18, 19).

Results

Since virus isolation from tumour cells, tumour cell extracts or separated T lymphocytes and their extracts in the case of 25 patients with various type of malignant urogenital tumours was unsuccessful, we attempted to isolate infectious viruses from PHA stimulated T lymphocytes and their extracts of additional 30 patients with malignant urogenital tumour. Infectious virus could be recovered in 1 case out of the 30, namely from the stimulated lymphocytes of a patient with bladder tumour.

Case history. Mrs Zs. B., 68 female, was admitted to our department with frequent micturition and haematuria. Intravesical ultrasonic examination revealed that on the right side of the bladder there was a tumour of solid echo-structure, spreading from the air sack to the base of the bladder, breaking through the wall and spreading over the surroundings (T₃b stage). As shown by cystoscopy, the tumour filled the right half of the bladder and spread over the anterior wall, and infiltrated the right orifice. Its surface was bullosus, oedematous, in some places covered with necrotic fibrin. The left half of the bladder and the left orifice were normal. Because of the advanced stage of the tumour, sectio alta was performed using electrocoagulation and electrosection. Microscopic examinations revealed a tumour of nidatory structure made up of urothel. The tumour cells were atypical. Diagnosis: carcinoma urotheliale invasum (St. T₃b).

Virological investigations. The separated and PHA-stimulated lymphocytes of the patient were placed upon cell cultures. No change was visible in the cells for weeks. After 7-8 weeks some swollen and ragged-edged cells, perhaps cell-focus etc., were appearing indicating the presence of a cytopathic agent. Then the cells were scraped off the wall of the culture vessel and placed

on fresh cell cultures where the number of virus indicating cells increased and in a few weeks the cytopathic effect became more and more expressed. When the material was placed on a new fresh HEp-2 and amniotic cell culture, the time of the development of the cytopathic effect shortened to 1 week. From that time onwards the unknown agent (virus) multiplied unrestrictedly. We produced a sufficient amount and started the identification of the virus.

In the examinations we used complement-fixing and agar gel precipitation methods with immune sera produced against different types of adeno- and herpes viruses. The new agent proved to belong to the group of adenoviruses. To determine the adenovirus type, we used 10 different adenovirus type specific immune sera. With the sera virus neutralization test was performed in tissue culture. The isolated virus did not react with sera against 9 types whereas one type of serum completely neutralized it. In the end the virus proved to be an adenovirus, type 18, of oncogenic character. DNA examination performed with Hind-III restriction enzyme confirmed that the virus belonged to the adenoviruses.

Discussion

Our previous studies showed that oncogenic adeno- and herpes viruses may be present in the tumour cells of patients with tumours in the form of their components or active genome [1]. We proved the presence of specific antibodies against the protein components of the virus [2]. Virus components were found also in a small per cent of the circulating lymphocytes of tumour patients [3]. However, so far we have not succeeded in detecting infectious viruses either in tumour cells or from lymphocytes. Thus it may be considered a significant fact that after PHA stimulation we managed to gain an infectious adenovirus belonging to oncogenic types from the circulating lymphocytes of a patient with progressive bladder tumour (T₃b stage).

The significance of the isolated virus is emphasized by the fact that in lymphocyte transformation tests performed since then the lymphocytes of patients with urological tumours mainly reacted with the new type of adenovirus (unpublished data). Thus, the genome may be supposed to play certain role in the development of the tumour in interaction with other DNA viruses and the damaged immune system.

At present there are intensive studies being carried out to survey and study the action of the genome of the adenoviruses [4-8]. The influence of several segments of the adenovirus gene on the function of immune system is known already [9-11]. Moreover, the results indicate that the oncogenic capacity of the adenoviruses, found in experimental circumstances, can appear in man mainly in interaction with other DNA viruses and also when the immune function changes [12-14]. In the case studied the immune functions of

the patient may have been weak due to the progressive tumour but in vitro under the influence of a strong mitogen the latent virus genomes in the cells were able to produce viruses of complete value.

REFERENCES

1. Kulcsár, G., Dán, P., Csata, S., Horváth, J., Nász, I., Verebélyi, A., Ongrádi, J.: *Acta Microbiol Hung* **30**, 199 (1983).
2. Kulcsár, G., Csata, S., Dán, P., Horváth, J., Nász, I., Ongrádi, J., Verebélyi, A.: *Magyar Onkológia* **25**, 181 (1981).
3. Csata, S., Kulcsár, G., Dán, P., Nász, I., Verebélyi, A.: *Acta Chir Hung* **28**, 321 (1987).
4. Berk, A.: *Ann Rev Genet* **20**, 45 (1986).
5. Horváth, J., Palkonyay, L., Weber, J.: *J Virol* **59**, 1989 (1986).
6. Hurwitz, D. R., Chinnadurai, G.: *Proc Natl Acad Sci USA* **82**, 163 (1985).
7. Kövesdi, I., Reichel, R., Nevins, J. R.: *Cell* **45**, 219 (1986).
8. Moran, E., Grodzicker, T., Roberts, R., Mathews, M. B., Zerler, B.: *J Virol* **57**, 765 (1986).
9. Cook, J. L., Lewis, A. M.: *Science* **224**, 612 (1984).
10. Hen, R., Borrelli, E., Chambon, P.: *Science* **230**, 1391 (1985).
11. Schrier, P. I., Bernards, R., Vaessen, R. T., Houweling, A., van der Eb, A. J.: *Nature* **305**, 771 (1983).
12. Everett, R. D., Dunlop, M.: *Nucleic Acids Res* **12**, 5969 (1984).
13. Feldman, L. T., Imperiale, M. J., Nevins, J. R.: *Proc Natl Acad Sci USA* **79**, 4952 (1982).
14. Routes, J. M., Cook, J. L.: *J Immunol* **142**, 4022 (1989).

CLOACIN TOLERANCE AND ASPECIFIC COLICIN INSENSITIVITY OF HUMAN *ESCHERICHIA COLI* STRAINS

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(Received August 13, 1990)

Of 182 wild-type human, aerobactin producer *Escherichia coli* strains 86.3% were insensitive to cloacin. All randomly chosen 51 strains were relatively cloacin tolerant. Cloacin tolerant strains were not considerably more sensitive to hydrophobic drugs than the cloacin sensitive descendant strains. Pathogenicity of the cloacin sensitive strains was significantly lower ($p < 0.05$) in intraperitoneal mice infection than that of the cloacin tolerant ones. Suggesting a new aspect of the uptake mechanism of colicins, cloacin tolerance was very frequently associated with an aspecific insensitivity to a broad spectrum of colicins.

It is known that the precondition of the inhibition effect of cloacin, a colicin-type protein produced by *Enterobacter cloacae* DF13 on *Escherichia coli* [1, 2], is the presence of a 74 kDal receptor protein located in the outer membrane. The receptor is identical with the receptor of Fe^{+++} -aerobactin [3–5]. It was presumable that the majority of the aerobactin (aer) producer, wild-type strains were cloacin sensitive, because the frequency of the spontaneous $p^{+}t^{-}$ mutants (aer producer, but defective in receptor) could not be high. As a consequence, the sensitivity to cloacin is used as an indicator of aer receptor by some authors [6, 7].

In the present work in contrast with the expectation, it was observed that the majority of the wild-type, human, aerobactin producer (aer^{+}) strains were not sensitive to cloacin. The possible explanation of this phenomenon and its connection with the sensitivity to other colicins was investigated.

Materials and methods

Bacterial strains. A total of 182 wild-type, human *E. coli* strains isolated in Hungary was examined. The strains were distributed into 4 groups: (a) multiple resistant strains isolated between 1979 and 1983 (they were stored freeze-dried till 1986 and in stock-agar afterwards); (b) strains carrying K1 capsular antigen, sensitive to antibiotics isolated in 1988 and 1989; (c) colicin producer strains, isolated in 1988; (d) strains, isolated in 1980 and stored in stock-agar. In addition, 11 col^{+} aerobactin producer and 19 col^{-} aerobactin non-producer laboratory strains were examined.

Media. Usually nutrient broth and agar was used. As iron-poor medium Simon–Tessmann agar [8] modified by Rabsch and Reissbrodt [9] supplemented with 160 μM dipyriddy and 20 $\mu g/ml$ nitriloacetic acid was used.

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Qualitative demonstration of aerobactin production was carried out by the biological assay of Carbonetti and Williams [6], modified by Rabsch and Reissbrodt [9] using *E. coli* LG1522 (L. Emödy, Institute of Microbiology, University Medical School, Pécs) as an indicator. Strains, giving positive reaction were designated aer⁺.

Cloacin solution. The supernatant of a 16 h nutrient broth culture of the cloacin producer *E. cloacae* DF13/N5160 strain (received from M. Dho, Station de Pathologie Aviaire et de la Parasitologie, Centre de Recherche de Tours, Institut National de la Recherche Agronomique, Nouzilly, Monnai, France) induced by mitomycin C (0.2 µg/ml) in the log phase was treated with chloroform. The 1 U/ml activity is the smallest quantity, which partially inhibits the growth of strain *E. coli* Tr 30-17 (M. Dho) in nutrient agar.

Determination of cloacin sensitivity. The qualitative test was carried out with a cloacin solution of concentration 1024 U/ml in nutrient agar. The degree of sensitivity was determined by spotting a halving serial dilution on nutrient agar.

Test for determining cloacin adsorption. Cells grown in iron-poor agar were suspended in physiological saline, unboiled and boiled for 15 min then incubated in cloacin solution (64-128 U/ml) for 2 h at 37 °C. The decrease of the bacteriostatic activity of the chloroform treated supernatant was determined in halving serial dilution on nutrient agar, using strain *E. coli* Tr 30-17.

Qualitative determination of colicin sensitivity was carried out as described by Milch and Gyenes [10]; the colicin producer colonies were treated by UV for 20 min. For colicin production, standard *E. coli* and *C. freundii* strains were used (received from G. Lebek, Institut für Hygiene und Medizinische Mikrobiologie, University of Bern) except in case of colicin E6; it was produced by a wild-type *Shigella sonnei* strain. The used colicins belonged to group "A" (A, E1, E2, E3, E6, K) and "B" (B, D, Ia, Ib, V) [11].

Quantitative determination of colicin sensitivity. The supernatants of 16 h shaken broth cultures of the colicin producer strains were induced by 0.2 µg/ml mitomycin C in the log phase (in case of colicin V mitomycin C was omitted). After chloroform treatment the MIC values were determined in U/ml, using ten-fold serial dilution on nutrient agar. The smallest quantity of colicin which inhibited the strain *E. coli* K12 was considered as 1 U.

Determination of colicin production was carried out according to Milch and Gyenes [10], colicin V was identified on the basis of the colicin immunity using the strains *E. coli* 711, 167-1, 59-5, Tr-41 and Tr-30-17 (received from M. Dho).

Phage typing. The method described by Milch and Gyenes [10] was used. The standard phage-set consisted of 30 phages and phage-pools.

Determination of LD₅₀ was carried out by intraperitoneal infection of mice, according to Czirák et al. [12].

Drug sensitivity. Agar diffusion method on nutrient agar served for determining MIC values of novobiocin (Hoechst), crystal violet (Reanal), methylene blue (Merck), nalidixic acid (Chinoïn), erythromycin (Sigma), rifampicin (Serva), EDTA (disodium salt of ethylene diamine-tetraacetic acid) (Reanal), sodium lauryl sulphate (Reanal), neomycin (Institute for Drug Research, Budapest).

Plasmid profile analysis. For the preparation of plasmid DNA, the method of Kado and Liu [13] was used; electrophoresis was carried out in vertical agarose gels according to Meyers et al. [14]. Standard plasmids for molecular size estimation: R1 (62 Mdal; N. Datta, Royal Postgraduate Medical School, London, U.K.), R27 (112 Mdal; N. Datta), pSP1 (140 Mdal; T. Pál, Institute of Microbiology, University Medical School, Pécs), V517 (35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4 Mdal [15]).

R plasmid transfer by conjugation. Two-hour broth cultures of the donor and recipient strains were mixed (1:1) and streaked to nutrient agar. After incubation at 37 °C for 16 h, recombinant colonies were selected on nutrient agar containing 20 µg/ml antibiotic adequate to the R plasmid markers.

Statistical analysis. The chi-square test was used.

Results

Table I presents the frequency of cloacin sensitivity of 182 human, wild-type, aer⁺ *E. coli* strains. The strains were cloacin sensitive only in 13.7%. The frequency of the sensitive strains showed significant differences in the

different groups (1.6–27.1%). In contrast with the wild-type strains, most *aer*⁺ laboratory strains were sensitive (10 out of 11). The MIC values of the wild-type *clo*^s strains varied in a wide range; higher values were detected than among the *clo*^s laboratory strains (Fig. 1).

Table I

Frequency of cloacin-sensitivity among wild-type, human, aer⁺ E. coli strains

Groups*	No. of strains	Cloacin sensitive strains	
		No.	%
a	70	19	27.1
b	29	2	6.9
c	19	3	15.8
d	64	1	1.6
Total	182	25	13.7

The strains were evaluated according to the bacteriostatic effect of a cloacin solution of 1024 U/ml concentration

* For characterization of groups see Materials and methods

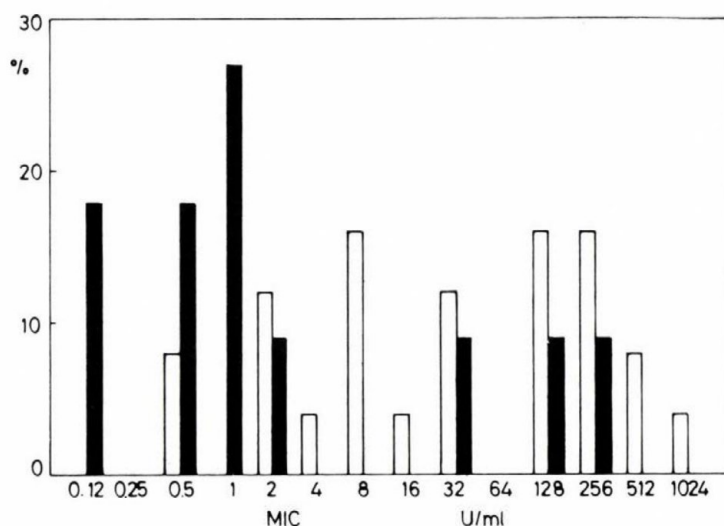


Fig. 1. Distribution of colicin-sensitive strains according to the degree of sensitivity. Open columns: 25 wild-type strains; solid columns: 11 laboratory strains

The cloacin adsorption of 51 random chosen *aer*⁺ cloacin insensitive strains belonging to the group (a) and (c) was examined. The activity of cloacin solution was reduced in all cases when it was incubated with the heat-treated cell-suspension. In contrast with these findings, no decrease was observed in case of the *aer*⁻ strains as compared to the cell-free incubation. From the

above mentioned strains 15 were randomly chosen and cultured on modified Simon-Tessmann agar not-containing dipyriddy chelator and nitriloacetic acid and complemented with ferric chloride. The decrease of activity of the cloacin solution effected by these cell suspensions was significantly less than by the cells grown on iron-poor agar. If originally aer⁺, cloacin insensitive strains, which lost their aerobactin producing capacity were used for incubation, their effect on the reduction of cloacin activity was also lost. This effect appeared in the transconjugant strains after the acquisition of the aer operon carrying R plasmids, by conjugation.

Table II shows the comparison of sensitivity of 4 pairs of strains to cloacin, 11 kinds of colicins and *E. coli* phage-set. Strain 981/b [16] was obtained after UV and mitomycin C treatment from a clo^{tol} strain 981/a, which was insensitive to 8 colicins in the used concentration; strain 981/b, while loosing its R plasmid became clo^s and simultaneously it became sensitive or more sensitive to some colicins (some of them belonged to colicin group "B"). The change of sensitivity was not connected with the loss of the 36 Mdal plasmid, because the sensitivity patterns of strains 981/c-d, which were derived from strains *a* and *b*, respectively, by the change of plasmid-profile (see text in Table II), reproduced the sensitivity patterns of strains *a* and *b*. Strain 75/a was a wild-type strain, stored for 6 years in lyophilized form and further on for 2 years on Dorset medium; it was clo^{tol} and was sensitive, though in a low degree, to a broad spectrum of colicins. Strain 75/b clo^s was

Table II
Colicin and phage sensitivity

Strains	Aerobactin production	Cloacin sensitivity MIC (U/ml)	Colicin sensitivity					
			"A"					
			A	E1	E2	E3	E6	K
981/a	+	tolerant	10 ³	10 ³	> 10 ⁴	> 10 ³	10 ⁴	> 10 ⁴
<i>b</i>	+	8	10 ¹	10 ¹	10 ¹	10 ¹	10 ¹	10 ¹
<i>c</i>	—	resistant	10 ²	10 ³	10 ²	> 10 ³	10 ³	> 10 ⁴
<i>d</i>	—	resistant	10 ¹	10 ¹	10 ¹	10 ¹	10 ¹	10 ¹
75/a	+	tolerant	10 ²	10 ²	10 ²	10 ²	10 ²	> 10 ⁴
<i>b</i>	+	128	10 ⁰	10 ⁰	10 ⁰	10 ⁰	10 ⁰	10 ⁰
674/a	+	128	10 ²	10 ¹	10 ³	10 ³	10 ⁴	10 ¹
<i>b</i>	+	8	10 ¹	10 ¹	10 ¹	10 ¹	10 ¹	10 ⁰

Origin of strain-pairs: 981/a: original strain; *b*, *c*: from strain *a*, by plasmid elimination by UV + mitomycin C treatment [16]; *d*: from strain *b*, first plasmid-free variant was made by selection with cloacin then transfer of the original R-plasmid was performed by conjugation. 75/a: original strain; *b*: from strain *a* by serial passages. 674/a and *b*: spontaneous variants obtained after serial passages

derived from the former strain by serial subculturing, during 1 year. Sensitivity to colicins increased aspecifically, including also to colicin group "B". Strains 674/a and 674/b were the spontaneous variants isolated from a common ancestor, both were also clo^s but in a different degree. The increased sensitivity to cloacin was associated with increased sensitivity to colicins, but only to the members of group "A". In the case of the first three pairs of strains the increase of colicin sensitivity was associated with the amplification of phage patterns. There was no change in case of strain-pair 674.

The MIC values of hydrophobic drugs and detergents were compared in case of the above mentioned pairs of strains by agar diffusion method (Table III). In case of pairs *E. coli* 981 and *E. coli* 75 the ratio of MIC values did not deviate from 1, or only within the scale. In case of pair 674 the variant a, which was less sensitive to cloacin proved to be more sensitive beyond the scale to more, but not to every drug. There was no correlation between the rate of deviation and the hydrophobicity characterized by the partition coefficient of the drugs.

Table IV shows the sensitivity spectrum to 11 colicins of numerous cloacin sensitive and tolerant strains, determined by qualitative test. It was found that more than 1/3 of the clo^{tol} strains were insensitive to all of the 11 colicins and only one strain was sensitive to more than 3 colicins. On the other hand, 80% of the wild-type clo^s strains and about 90% of the laboratory strains were sensitive at least to 4 colicins.

of *E. coli* strain-pairs

MIC U/ml					0 antigen*	Plasmid profile	Phage pattern	Colicin production
“B”								
B	D	Ia	Ib	V				
10 ⁴	> 10 ⁴	> 10 ²	> 10 ¹	> 10 ²	078	36+ 78++	3, 4a, 4b	V
10 ¹	10 ¹	> 10 ²	> 10 ¹	> 10 ²	078	78++	3, 4a, 4b, 7	V
10 ⁴	> 10 ⁴	> 10 ²	> 10 ¹	10 ²	078	36+	3, 4a, 4b	—
10 ¹	10 ¹	> 10 ²	> 10 ¹	> 10 ²	078	36+	3, 4a, 4b, 7	—
10 ⁴	10 ⁴	10 ²	> 10 ¹	10 ²	(0141)	3,2 38	—	—
10 ⁰	10 ⁰	10 ⁰	10 ⁰	10 ⁰	(0141)	3,2 38	4, 6	—
10 ⁴	10 ⁴	10 ²	10 ¹	> 10 ²	0143	1,8 89	—	V
10 ⁴	10 ⁴	> 10 ²	> 10 ¹	> 10 ²	(0143)	1,8 89	—	V

* In parenthesis: the O antigen of the original strain determined at isolation could not be demonstrated by tube agglutination test during the experimental period

+ R-plasmid

++ Col V plasmid carrying aer operon

Table III

Sensitivity of E. coli strain-pairs to hydrophobic drugs

Substance	Partition coefficient	MIC ratios		
		75b(clo ^s)/ 75a(clo ^{tol})	981b(clo ^s)/ 981b(clo ^{tol})	674b(clo ^s)/ 674a(clo ^s)
Novobiocin	20***	4	4	64
Crystalviolet	14.4*	1	1	16
Chloramphenicol	12.4*	1	NT ⁺	8
Rifampicin	8.8***	1	1	2
Nalidixic acid	3.16**	1	1	4
Erythromycin	0.79**	1	0.5	16
Methylene blue	0.02**	1	1	16
Sodium lauryl sulphate	0.02**	1	4	64
EDTA-Na ₂	—	1	1	1
Neomycin	0.01*	1	1	1

MIC values were determined by agar-diffusion method on nutrient agar

⁺ Not tested (strain 981/a carried an R-plasmid determining chloramphenicol, tetracycline, ampicillin resistance)

Partition coefficients: according to Nikaido* [17], Coleman and Leive** [18], Osada and Beppu*** [19]

Table IV

Distribution of E. coli strains according to the breadth of colicin-sensitivity spectrum

Phenotype of strains			No. of strains	Strains sensitive to			
				0	1-3	4-8	9-11
				colicins			
Wild-type aer ⁺	clo ^{tol} col ⁻	22	11 (50.0)	11 (50.0)	0	0	
	clo ^{tol} col ⁺	29	8 (27.5)	20 (68.9)	1 (3.4)	0	
	clo ^{tol} total	51	19 (37.2)	31 (60.7)	1 (1.9)	0	
	clo ^s col ⁻	13	0	3 (23.0)	4 (30.7)	6 (46.1)	
	clo ^s col ⁺	12	0	2 (16.6)	10 (83.3)	0	
	clo ^s total	25	0	5 (20.0)	14 (56.0)	6 (24.0)	
	Laboratory strains	aer ⁻ clo ^r col ⁻	19	0	2 (10.5)	3 (15.7)	14 (73.6)
aer ⁺ clo ^s col ⁺		9	0	1 (11.1)	3 (33.3)	5 (55.5)	
total		28	0	3 (10.7)	6 (21.4)	19 (67.8)	

In parentheses: percentages

Figure 2 presents the data of Table IV grouped according to the efficacy of colicins. The tendency was obvious both in case of col⁻ and col⁺ wild-type aer⁺ strains: the colicins were more effective to clo^s strains than to clo^{tol} ones. In case of col⁻ strains, the average effectiveness of the two colicin-groups increased nearly in the same extent. For col⁺ strains, which produced

colicin V too, the increase of the efficacy was less in group "B". The efficacy of both colicin groups was almost identical (i.e. higher than 80%) for col⁻ laboratory strains. Among the wild-type clo^s strains, the increased sensitivity to cloacin tended to be associated with a wider spectrum of colicin sensitivity. Dividing the strains into two groups on the basis of the range of the colicin sensitivity spectrum, it was found that in the group, sensitive to 9–11 colicins the average cloacin sensitivity (MIC) was 8 U/ml while in the group, sensitive to 2–8 colicins, the average MIC was 128 U/ml.

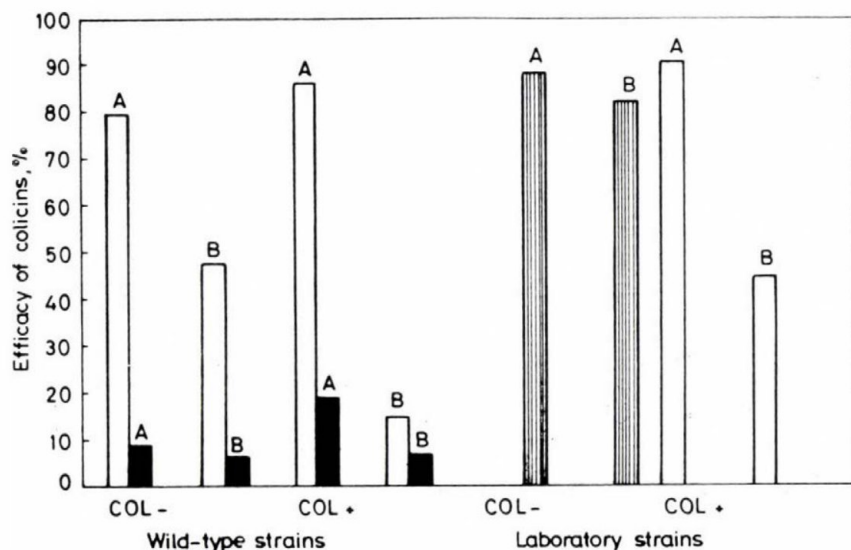


Fig. 2. Effectiveness of colicin-groups "A" and "B" to clo^{tol} and clo^s strains. Colicins: group "A": A, E1, E2, E3, E6, K; group "B": B, D, Ia, Ib, V. Wild-type aer⁺ strains: 25 clo^s (13 col⁻, 12 col⁺) strains; 51 clo^{tol} (22 col⁻, 29 col⁺) strains. Laboratory strains: 19 aer⁻ clo^s col⁻, 11 aer⁺ clo^s col⁺ strains. Calculation of efficacy of colicins: the total number of inhibiting effects within a given strain-, and colicin-group compared to the possible maximal number of inhibitions (number of strains $\times$ number of colicins) expressed in percentage. Open columns: clo^s; solid columns: clo^{tol}; shaded columns: clo^r.

In Table V the LD₅₀ values are presented, carried out with the strains of group (a), by intraperitoneal mouse infection. All the LD₅₀ values of the clo^s strains were 10⁶ or greater but these values were less than 10⁶ among the 23% of the clo^{tol} strains.

Discussion

Cloacin was absorbed not only by cloacin sensitive but also by cloacin insensitive aer⁺ strains which included nearly the 90% of the wild-type aer⁺ strains. The loss of aer production resulted in the loss of adsorption, but it

reappeared after the acquisition of the plasmid mediating aerobactin production. Since a possible aspecific adsorption does not depend on the presence or the activity of the aer operon, cloacin adsorption of the insensitive strains must take place in the specific receptors. Therefore, these strains can be classified as tolerant to cloacin in the used concentration. This is supported also by the repression of adsorption by iron.

The cloacin binding capacity of the sensitive strains was not higher than that of the tolerant starting strains (unpublished data). Therefore, the masking effect of S-LPS on the receptors, which was responsible for the insensitivity

Table V

Pathogenicity of cloacin-sensitive and tolerant strains in i.p. infected mice

Phenotype \ LD ₅₀	n.10 ⁷		n.10 ⁶		n.10 ⁵		Total
	No.	%	No.	%	No.	%	
Clo ^s	10	52.6	9	47.4	0	0	19
Clo ^{toi}	18	35.3	21	41.2	12	23.5	51
Total	28	40.0	30	42.8	12	17.2	70

Both phenotypic groups were distributed into less ($LD_{50} \geq 10^6$) and more ($LD_{50} < 10^6$) pathogenic subgroups. The frequency of the latter subgroup differed significantly ($p < 0.05$) between the two phenotypes

to colicins in several cases [7, 20, 21], does not give an explanation on cloacin tolerance.

Because of the frequency of the cloacin tolerant, wild-type human strains, cloacin insensitivity is not an evidence for the absence of the aer receptor.

Whereas, tolerance to "E"-type colicins has been described, to our knowledge tolerance to cloacin is a new finding. Though cloacin DF13 being the last member of an evolutionary line [22] is in close relationship with "E" colicins, and its mechanism of action is the same as that of E3 (a 16S rRNA degrading enzyme [2, 23]), the two substances are not identical. They differ not only in their molecular sizes, but the sequences responsible for binding are not homologous [22], in accordance with the fact that colicins E are bound to the *btuB* receptor, but the common receptor for cloacin and aerobactin is coded by the gene *iutA* [5]. Therefore, tolerance to some E-type colicins does not mean tolerance to cloacin as well.

Akutsu et al. [22] described that cross-immunity exists between cloacin and colicin E6. Cloacin tolerance among the strains examined by us cannot

be explained by cross-immunity, because the clo^{tol} strains were col^- in 43%. According to publications on the mechanism of tolerance to E-type colicins, partly proteins of transport function and of the outer membrane, partly the mutations of LPS are responsible.

The proteins coded by the *tolQRAB* genes, localized in the cytoplasmic membrane and periplasm are required for the translocation from the receptor [24–27]. *TolC* mutations [28–32] responsible for E1 tolerance were supposed to be in the outer membrane. All these mutations increased sensitivity to antibacterial materials of hydrophobic character in a pleiotropic manner. According to Eriksson-Grennberg and Nordström [33] and Osada and Beppu [19] the changes in the sugar content of LPS are responsible for the E2–E3 and E2 tolerance. The increased sensitivity to hydrophobic materials were also demonstrated by the latter authors.

In the phenotypes *tol* IV and *tol* XIV described by Davies and Reeves [34] and in case of mutations *tolF* [35, 36] and *tolG* [37] changes took place in the composition of the outer membrane proteins. But in these cases tolerance did not extend to colicin group “B”. A relatively aspecific tolerance to colicin A, E2, K, L, N and S4 was caused by envelope mutation as demonstrated by Davies and Reeves [34], but all these colicins belonged to group A.

In case of our $\text{clo}^- \text{clo}^5$ strain pairs only slight differences were observed in sensitivity to hydrophobic drugs in the two examined sensitive-tolerant strain pairs (Table III) and tolerance was associated with a relative insensitivity to several colicins in an aspecific manner (which depended neither on immunity, nor on a transport system specific to a colicin-group), since it extended to the representatives of both groups.

The almost 90% frequency of cloacin insensitivity of the wild-type strains (which means tolerance to cloacin to all probability) and the statistical correlation of cloacin tolerance and the broad spectrum of colicin insensitivity (Table IV, Fig. 2) supports our opinion that the observed phenomenon cannot be explained by the known mutations. It may be assumed that a barrier exists in the bacterial envelope (most probably in the outer membrane), which can inhibit in an aspecific manner the translocation of the cloacin from the receptor, though all the necessary gene products according to the literature may be present. Depending on the expression of this barrier, cloacin-sensitivity of different degree or insensitivity to the given cloacin concentrations may appear.

In case of strain-pair 674 the phenomenon may be similar to that observed by the *tolQRAB* mutants, because the strain less sensitive to cloacin showed an extreme sensitivity to some hydrophobic materials (Table III) comparing to the one which was more sensitive to cloacin. Furthermore, while the sensitivity to cloacin-group “A” increased (except colicin E1) simultaneously with colicin sensitivity, it did not increase to colicin-group “B” (Table II).

The frequency of the low LD₅₀ values was significantly higher among the clo^{tol} strains, than among the clo^s ones (Table V). Cloacin sensitivity, occurring rarely among the wild-type strains *in vivo* or during serial passages can serve as an indicator of the beginning of some degradation of LPS decreasing the barrier of cloacin translocation and pathogenicity. This degradation may be S-R variation with or without any other changes of LPS. It is in accordance with this supposition that the less virulent, R-type laboratory strains were sensitive to cloacin in almost 100% when the receptor was present and their colicin-sensitivity spectra were very wide.

It is also possible that the barrier function is in connection with LPS-protein interaction. In case of colicin K the *tsx* receptor must be in functional interaction with the LPS and with the *ompA* protein for the translocation from the receptor [38]. It was supposed by Hinz [39] as one of the explanations that the *tsx-ompA* interaction causes a local disturbance in the outer membrane, so its barrier function can decrease. During the development of the general colicin sensitivity in our case the outer membrane may be loosened by such disturbances.

The explanation for the observed frequency of cloacin-sensitivity of group "A" (27.1%) may be that these strains have been subcultured several times during our previous work and these strains form a transition to laboratory strains.

The significance of the colicin production in the *in vivo* competition among the strains may be that it acts as a selective factor against the colicin sensitive mutants of decreased virulence.

We would like to prove our hypothesis by SDS-PAGE analysis of the LPS and outer membrane protein content of the isogenic strain-pairs; these experiments are in progress.

REFERENCES

1. Tieze, G. A., Stouthamer, A. H., Jansz, H. S., Zandberg, J., van Bruggen, E. F. J.: *Mol Gen Genet* **106**, 48 (1969).
2. De Graaf, F. K., Spanjaardt-Speckman, E. A., Stouthamer, A. H.: *Antonie van Leeuwenhoek* **35**, 287 (1969).
3. Bindereif, A., Braun, V., Hantke, K.: *J Bacteriol* **150**, 1472 (1982).
4. Grewal, K. K., Warner, P. J., Williams, P. H.: *FEBS Lett* **140**, 27 (1982).
5. Van Tiel-Menkvel, G. J., Mentjox-Vervuurt, J. M., Oudega, B., de Graaf, F. K.: *J Bacteriol* **150**, 490 (1982).
6. Carbonetti, N. C., Williams, P. H.: *Infect Immun* **46**, 7 (1984).
7. Lafont, J. P., Dho, M., D'Hauteville, H. M., Bree, A., Sansonetti, P. J.: *Infect Immun* **55**, 193 (1987).
8. Simon, E. H., Tessmann, J.: *Proc Natl Acad Sci USA* **50**, 526 (1963).
9. Rabsch, W., Reissbrodt, R.: *J Basic Microbiol* **25**, 663 (1985).
10. Milch, H., Gyenes, M.: *Acta Microbiol Hung* **19**, 213 (1972).
11. Davies, J. K., Reeves, P.: *J Bacteriol* **123**, 96 (1975).
12. Czirók, É., Milch, H., Vincze, I.: *Acta Microbiol Hung* **34**, 25 (1987).
13. Kado, C. I., Liu, S.-T.: *J Bacteriol* **145**, 1365 (1981).
14. Meyers, J. A., Sanchez, D., Elwell, L. P., Falkow, S.: *J Bacteriol* **127**, 1529 (1976).

15. Macrina, F. L., Kopecko, D. J., Jones, K. R., Ayers, D. J., McCowen, S. M.: Plasmid **1**, 417 (1978).
16. Milch, H., Nikolnikov, S., Cziráková, É.: *Acta Microbiol Hung* **31**, 117 (1984).
17. Nikaido, H.: *Biochim Biophys Acta* **433**, 118 (1976).
18. Coleman, W. G. Jr., Leive, L.: *J Bacteriol* **139**, 899 (1979).
19. Osada, H., Beppu, T.: *Agr Biol Chem* **49**, 1813 (1985).
20. Derbyshire, P., Baldwin, T., Stevenson, P., Griffiths, E., Roberts, M., Williams, P., Hale, T. L., Formal, S. B.: *Infect Immun* **57**, 2794 (1989).
21. Van der Ley, P., de Graaff, P., Tommassen, J.: *J Bacteriol* **168**, 449 (1986).
22. Akutsu, A., Masaki, H., Ohta, T.: *J Bacteriol* **171**, 6430 (1989).
23. De Graaf, F. K.: *Zentralbl Bakteriell (A)* **244**, 121 (1979).
24. Bernstein, A., Rolfe, B., Onodera, K.: *J Bacteriol* **112**, 74 (1972).
25. Braun, V.: *J Bacteriol* **171**, 6387 (1989).
26. Levengood, S. K., Webster, R. E.: *J Bacteriol* **171**, 6600 (1989).
27. Sun, T.-P., Webster, R. E.: *J Bacteriol* **165**, 107 (1986).
28. Nagel de Zwaig, R., Luria, S. E.: *J Bacteriol* **99**, 78 (1969).
29. Nomura, M.: *Ann Rev Microbiol* **21**, 257 (1967).
30. Luria, S. E.: In Leive, L. (ed.): *Bacterial Membranes and Walls*. Marcel Dekker Inc., New York 1973, p. 293.
31. Otsuji, N., Higashi, T., Kawamata, J.: *Biken J* **15**, 49 (1972).
32. Morona, R., Reeves, P.: *J Bacteriol* **150**, 1016 (1982).
33. Eriksson-Grennberg, K. G., Nordström, K.: *J Bacteriol* **115**, 1219 (1973).
34. Davies, J. K., Reeves, P.: *J Bacteriol* **123**, 102 (1975).
35. Chai, T. J., Foulds, J.: *J Bacteriol* **130**, 781 (1977).
36. Bassford, Ph. J. Jr., Diedrich, D. L., Schnaitman, C. L., Reeves, P.: *J Bacteriol* **131**, 608 (1977).
37. Chai, T. J., Foulds, J.: *J Mol Biol* **85**, 465 (1974).
38. Brass, J. M.: *Curr Top Microbiol Immun* **129**, 1 (1986).
39. Hinz, U.: Ph D Thesis. University of Basel, Switzerland 1983.

RESPONSE OF *ESCHERICHIA COLI* STRAINS CARRYING PLASMID(S) AND THEIR PLASMIDLESS DERIVATIVES TO BACTERICIDAL ACTIVITY OF HUMAN SERUM AND POLYMORPHONUCLEAR LEUKOCYTES

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(Received August 18, 1990)

Ten virulence plasmid(s) and antibiotic resistance plasmid(s) carrying *Escherichia coli* strains isolated from stool of infants with diarrhoea and their plasmidless derivatives were examined for response to bactericidal activity of human serum and intracellular killing of polymorphonuclear leukocytes (PMN). Plasmid(s) carrying isolates exhibited a significantly higher resistance to serum and phagocytosis.

It is known that result of interaction between microorganisms and host is influenced by the virulence properties of invading bacteria on one hand and ability of the host to avoid infection on the other hand. In *Escherichia coli* strains some properties has been suggested to be important in virulence, e.g. some O and K antigens, adhesins, enterotoxins, aerobactin, alpha-haemolysin [1–3]. Also resistance of bacteria to serum bactericidal activity and phagocytosis has been suggested [4].

Virulence properties in *E. coli* strains may be encoded not only chromosomally, but by plasmids as well. This may be very important because plasmids may give bacteria genetic flexibility permitting them a rapid spread of the pathogenic traits through bacterial populations [5]. Moreover, it is known that plasmid-mediated properties could be involved in the resistance of *E. coli* strains to human serum bactericidal activity and to phagocytosis [6].

Investigating the ability of *E. coli* strains to resist to bactericidal activity of human serum and to intracellular killing of PMN, we have recently used pooled serum and PMN both isolated from various blood donors. Response of bacteria to serum and intracellular killing of PMN was considerably different [7]. To eliminate differences depending on possible function variances in donors and to evaluate better the influence of a single virulence trait to bacterial serum resistance and resistance to intracellular killing of PMN, blood from one healthy donor was used in this work.

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Materials and methods

Bacterial strains. Ten *E. coli* strains isolated from stool of infants with diarrhoea were used. The strains were serogrouped by using eight polyvalent and fifty two monovalent O antisera (Imuna).

Serum. Blood from one healthy donor was obtained by venipuncture and allowed to clot for 15 min at room temperature. After centrifugation at 1500 g the serum was removed, fractionated into small volumes and stored at -70°C until needed.

Phenotype. Presence of fimbriae was determined by testing mannose-resistant and mannose-sensitive haemagglutination of human, bovine, chicken and guinea pig erythrocytes [8]. Production of heat-stable and heat labile enterotoxin was determined by using the Biken test [9]. Production of alpha-haemolysin was shown after supplementing of supernatants of strains grown on alkaline meat extract broth by bovine erythrocytes [10]. Colicin production was tested by the double-layered agar method. *E. coli* ROW sensitive to all colicins was used as indicator strain. For determination of colicin V production a mutant *E. coli* strain ROW resistant to colicin V was used [11, 12]. Agar dilution plate method was used for determination of resistance to six antimicrobial agents: tetracycline (Tc), streptomycin (Sm), chloramphenicol (Cm), kanamycin (Km), ampicillin (Ap), sulphonamide (Su). Transfer of resistance was determined in simple conjugation test employing *E. coli* C 600 Rif as recipient strain [13].

Genotype. Plasmids were extracted by the alkaline lysis method [14] and lysates were subjected to agarose 0.7% gel electrophoresis with TAE buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA, pH 7.6). Gels were stained in TAE buffer containing ethidium bromide (0.5 µg/ml) for 40 min and destained in distilled water for 10 min before visualisation under UV illumination.

Plasmid curing was done using acridine orange stain. Briefly, fewer than 1000 c.f.u./ml was inoculated into 5 ml of L-broth containing 5 µg of acridine orange per ml. Incubation was continued for up to 96 h [15].

Serum bactericidal assay. The serum bactericidal assay was modified slightly from a previously described method [16]. For these experiments overnight cultures of *E. coli* strains grown at 37°C on blood agar were harvested. The cells were adjusted in Hanks balanced salt solution (HBSS) to contain 2.5×10^4 c.f.u. in 1 ml. Microplates (Okula) were employed for incubation of bacteria with serum. One hole contained 0.05 ml of bacteria suspension (2.5×10^4 /ml) and 0.05 ml of 10% or 100% serum. The control hole contained 0.05 ml of HBSS instead of serum. The microplate was sealed with adhesive tape to prevent evaporation and placed on a roller (angle 45°) and rotated for 180 min. Samples (10 µl) were seeded after 60, 120 and 180 min incubation at 37°C on blood agar plates. The plates were incubated for 18 h at 37°C and colony counts were determined. Response of bacteria to serum bactericidal activity was expressed as percentage of surviving bacteria after 180 min related to the original count of bacteria found at $t = 0$ min in the controls. Strains were termed serum sensitive if the viable count dropped to less than 1% of the initial value and serum resistant if more than 90% of organisms survived after 180 min. Strains that gave results between these values were of intermediate sensitivity [17].

Phagocytosis assay. The intracellular killing of bacteria by human PMN was determined microscopically employing slightly modified acridine orange staining method [18]. Briefly, 0.2 ml of fresh blood was placed on sterile cover slip in humidified plastic chamber and allowed to clot for 60 min at 37°C . Coagulated blood was removed by rinsing the cover slips gently in warm (37°C) HBSS. Bacteria preopsonized in 100% serum at 37°C for 15 min were placed onto the PMN monolayer. After 60 min incubation the cover slips were removed, drip dried and stained with acridine orange (1 mg/ml) for 1 min (Figs 1A, B). In this assay, intracellular bacteria fluoresce green when viable and red if non-viable. A total of 100 PMN were counted. The percentage of killed bacteria was estimated as follows:

$$\text{per cent killed} = \frac{\text{total number of non-viable bacteria in 100 PMN}}{\text{total number of intracellular bacteria}} \times 100$$

For statistical estimation Student's *t* test was used.

Results

Only four of our isolates were classified with the O antisera available. Two of them belonged to serogroup O78 (No. 688, 693) one to O8 (No. 691) and one to O25 (No. 694). Six remaining isolates were untypable. Five strains were fimbriated (No. 688, 693, 694, 696, 697). In four of them alpha-haemolysin (No. 688, 694, 698, 699) and colicin (No. 689, 693, 696, 699) production was demonstrated. The isolates were resistant to two to six antimicrobial agents.

Table I

Characteristics of Escherichia coli strains isolated from stool of infants

Strain No.	Sero-group	Virulence factors ^a				Antibacterial agents resistance ^b					
		F	Hly	Col	Ent	Tc	Sm	Cm	Km	Ap	Su
1/688	078	+	+			Tc	Sm	—	—	—	Su
2/689	NT			+		Tc	Sm	—	—	Ap	Su
3/690	NT					Tc	Sm	Cm	—	Ap	Su
4/691	08					Tc	Sm	Cm	Km	Ap	Su
5/693	078	+		+		Tc	Sm	Cm	Km	Ap	Su
6/694	025	+	+			Tc	Sm	Cm	Km	Ap	Su
7/696	NT	+		+		Tc	Sm	—	—	—	—
8/697	NT	+				Tc	Sm	—	—	—	—
9/698	NT		+			Tc	Sm	—	—	Ap	Su
10/699	NT		+	+		Tc	Sm	—	—	—	Su

^a F = fimbriae, Hly = alpha-haemolysin, Col = colicin, Ent = enterotoxin

^b Tc = tetracycline, Sm = streptomycin, Cm = chloramphenicol,

Km = kanamycin, Ap = ampicillin, Su = sulphonamide

NT = nontypable

Squared antibacterial agents: ability to transfer antibiotic resistance determinant(s)

Resistance to tetracycline and resistance to streptomycin was shown in all isolates, moreover, in three of them (No. 689, 693, 697) the resistance was transferable. Isolate No. 689 exhibited, in addition, a transferable resistance to ampicillin and sulphamethoxydin, whereas No. 690 only to ampicillin (Table I).

Plasmid DNA was demonstrated in all isolates. The majority of plasmids visualized were of the size of 23 kilobase and more. Other plasmids ranged from 9 to 2 kilobase. In four isolates (No. 688, 694, 696, 698) one plasmid DNA was found compared to strain No. 691 in the lysate of which ten plasmid DNA bands were visualized. Moreover, in No. 690 lysate seven, in No. 689 and 693 lysates six plasmid DNA bands were demonstrated. Furthermore, in No. 697 lysate there were five plasmid DNA bands (Table II, Fig. 2). After

acridine orange treatment of plasmid(s) carrying isolates, plasmid DNA was not demonstrated in any of the lysates (Fig. 3).

As to interaction of plasmid(s) carrying isolates with 10% serum it was found that after 3 h eight of them showed serum resistance (No. 688, 689, 690, 694, 696, 697, 699) and the remaining two (No. 691, 693) showed inter-

Table II
Plasmid profiles in ten E. coli strains

Isolate No.	Number of plasmids of approximate size (kb)						
	>23	~23	~9	~6.5	~4.3	~2	<2
1/688	1						
2/689	2	1	1		2		
3/690	3	1		1	1	1	
4/691	3	1	1	2	1	2	
5/693	3	1		2			
6/694	1						
7/696	1						
8/697	3	1	1				
9/698	1						
10/699	2	1					

Table III
Response to 10% normal human serum of E. coli strains isolated from stool of infants

Strain No.	Plasmid(s) carrying E. coli strain			Plasmidless E. coli strain		
	per cent		survival after	hours		
	1	2	3	1	2	3
1/688	105	163	707	129	78	113
2/689	83	222	637	109	126	110
3/690	18	48	187	98	95	89
4/691	10	20	57	22	32	114
5/693	5	2	2	92	97	97
6/694	107	345	593	101	105	114
7/696	139	161	1001	108	101	106
8/697	14	78	307	82	99	93
9/698	38	215	306	72	97	88
10/699	70	202	549	68	101	96

mediate sensitivity. Compared to plasmid(s) carrying isolates in the same plasmidless derivatives after 3 h serum treatment, also eight isolate derivatives showed serum resistance (No. 688, 689, 691, 693, 694, 696, 697, 699) and two were of intermediate sensitivity (No. 690, 698). However, when survival of plasmid(s) carrying isolates and plasmidless derivatives was compared after 3 h 10% serum treatment of bacterial survival the latter exhibited significantly lower values ($p < 0.01$, Table III).

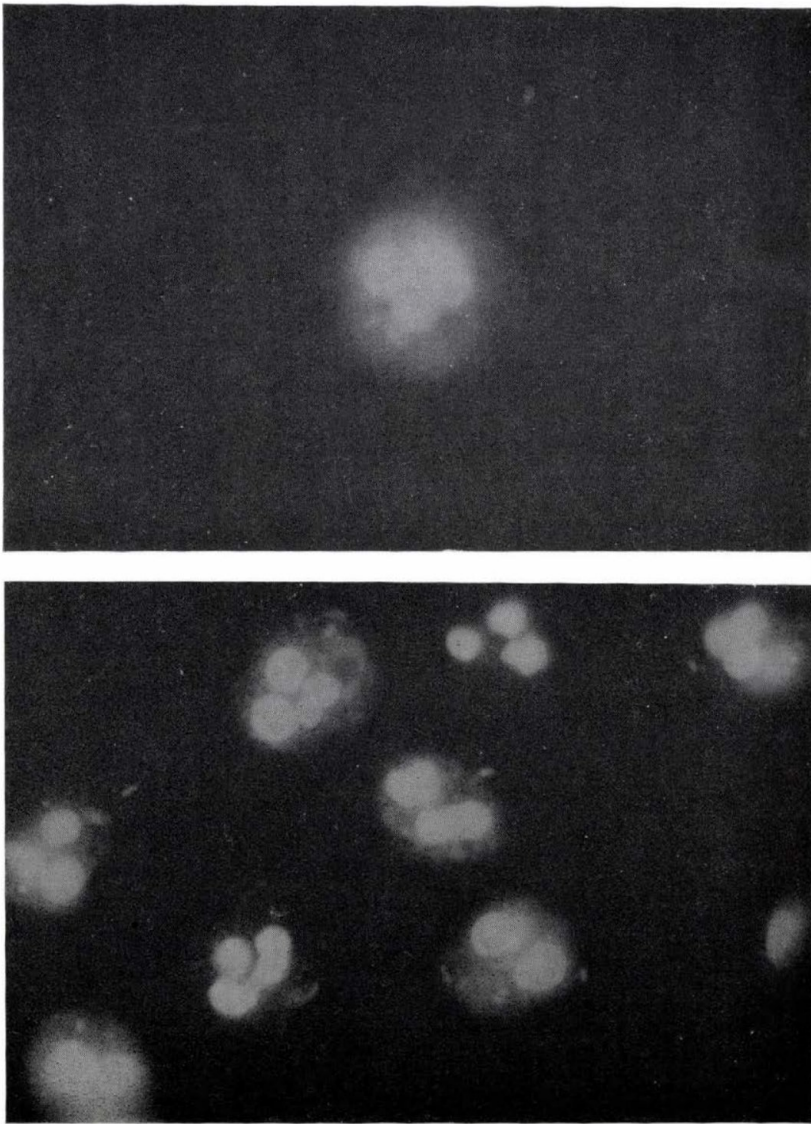


Fig. 1. A and B. Phagocytosis of *E. coli* by human PMN. Acridine orange staining. Under the microscope viable bacteria show green or orange, non-viable bacteria red fluorescence

More expressed differences in the survival of bacteria are listed in Table IV. Five of plasmid(s) carrying isolates showed serum resistance, another three were of intermediate sensitivity and remaining two were sensitive after 3 h concentrated serum treatment. Compared to plasmid(s) carrying isolates, none of plasmidless derivatives were serum resistant, another three were of intermediate sensitivity and the remaining ones were sensitive. When the

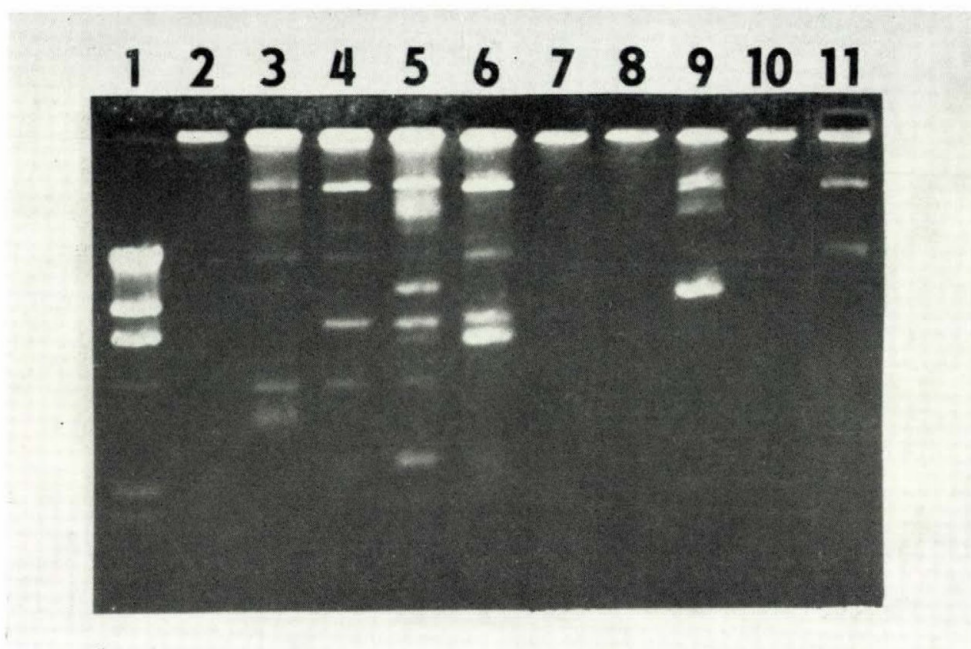


Fig. 2. Lane 1: a Hind III digest of lambda bacteriophage used as a molecular weight marker; lanes 2-11: plasmid profiles in *E. coli* strains No. 688, 689, 690, 691, 693, 694, 696, 697, 698, 699 from left to right

Table IV

Response to undiluted normal human serum of E. coli strains isolated from stool of infants

Strain No.	Plasmid(s) carrying <i>E. coli</i> strain			Plasmidless <i>E. coli</i> strain		
	per cent		survival	after	hours	
	1	2	3	1	2	3
1/688	92	114	220	31	0	0
2/689	92	129	382	82	0	0
3/690	14	1	0	76	4	<1
4/691	6	10	36	2	2	10
5/693	2	1	0	54	1	<1
6/694	110	133	197	85	10	3
7/696	77	93	11	62	6	0
8/697	33	43	17	51	4	<1
9/698	38	78	124	43	4	<1
10/699	78	72	96	51	7	2

survival of plasmid(s) carrying isolates and plasmidless derivatives was statistically evaluated, the latter exhibited significantly lower values of survival after 3 h concentrated serum treatment ($p < 0.05$).

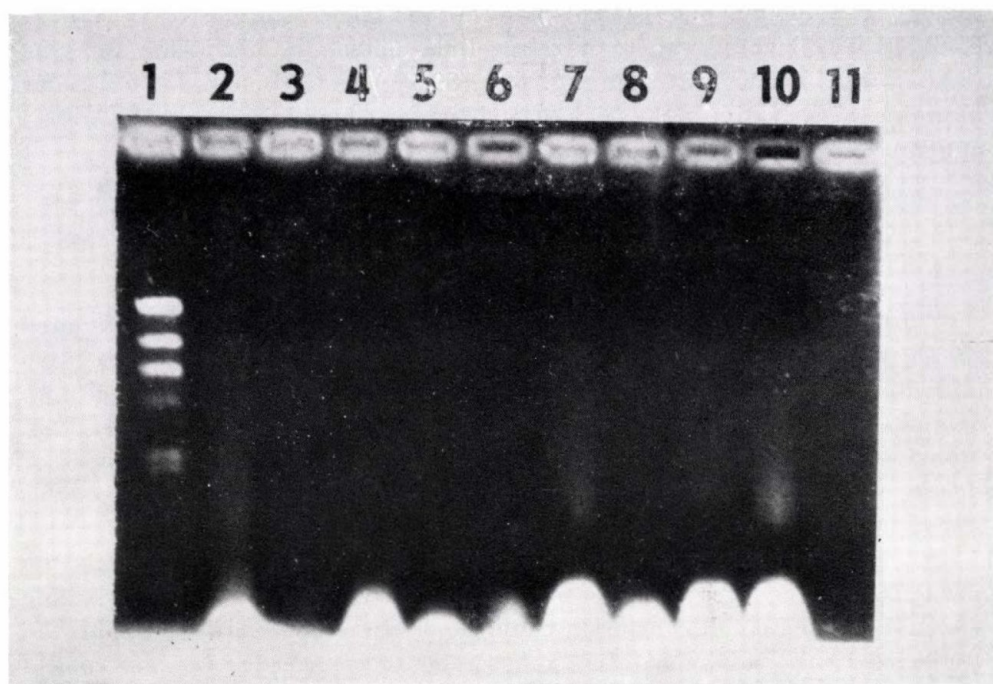


Fig. 3. Lane 1: a Hind III digest of lambda bacteriophage used as a molecular weight marker; lanes 2-11: extracted and visualized plasmid DNA from acridine orange treated *E. coli* strains No. 688, 689, 690, 691, 693, 694, 696, 697, 698, 699 from left to right

Table V

*Intracellular killing of E. coli strains
in human polymorphonuclear leukocytes*

Isolate No.	Per cent killed	
	Plasmid(s) carrying <i>E. coli</i> strain	Plasmidless <i>E. coli</i> strain
1/688	71	83
2/689	77	87
3/690	80.5	95
4/691	89	89
5/693	78	92.5
6/694	63.5	74
7/696	81.5	87.5
8/697	89.5	94.5
9/698	72.5	77
10/699	80	90.5

In plasmid(s) carrying isolates the average value of intracellular killing of bacteria in PNN was 78.2%, whereas in plasmidless derivatives 87% ($p < 0.05$). For plasmid(s) carrying isolates the lowest value of intracellular

killing was found in No. 694 (63.5%) and for plasmidless derivatives in No. 694 (74.0%). The highest values of intracellular killing in plasmid(s) carrying isolates were demonstrated in No. 697 (89.5%) and No. 691 (89.0%). For plasmidless derivatives, the highest values were found in No. 690 (95.0%) and No. 697 (94.5%) (Table V).

Discussion

In most publications serum resistance was examined comparing unrelated serum resistant and serum sensitive strains or inducing resistance in sensitive laboratory strains by gene transfer [19–21]. Therefore in the present study we compared pairs of wildtype *E. coli* strains and its plasmidless derivatives. Moreover, both serum and PMN were taken from one and the same healthy donor to prevent possible age-depending function variances of phagocytes shown by Sondell et al. [22] in healthy blood donors.

When results of response of plasmid(s) carrying *E. coli* strains and its plasmidless derivatives to serum bactericidal activity and intracellular killing of PMN are compared, conclusion could be drawn as to whether plasmids are conferred to the resistance of *E. coli* strains to nonspecific immune mechanisms. Similarly, the role of plasmid conferring effect to resistance of bacteria to serum and phagocytosis has been suggested by others [23–25]. However, from our results it is difficult to conclude exactly to which characteristic a greater importance upon interaction of bacteria with nonspecific immune mechanisms could be ascribed. *E. coli* strains carrying the most plasmids showed different response to serum and phagocytosis. *E. g.* isolate No. 691 carrying 10 plasmids showed intermediate sensitivity after concentrated serum treatment and a higher intracellular killing in PMN, but isolate No. 689 carrying 6 plasmids exhibited the highest serum resistance and at the same time a lower intracellular killing in PMN (Table V). It is noteworthy that these two isolates expressed different phenotype: isolate No. 691 was resistant to all antimicrobial agents used, while No. 689 with exception of transferable resistance to four antimicrobial agents produced colicin as well (Table I).

It has been reported that alpha-haemolysin contributes to the virulence of *E. coli* strains [26–28]. Therefore we focused attention to isolates No. 688, 694, 698 and 699 producing alpha-haemolysin. As it can be seen in Table IV, alpha-haemolysin producing *E. coli* strains were more resistant to serum than non-haemolytic ones. Our results are in accordance with those of Hughes et al. [29], who demonstrated association of haemolysin production with high levels of serum resistance in *E. coli* strains isolated from urinary tract infections. Similarly, Kubens and Opferkuch [30] found that loss of haemolysin secretion in a wildtype *E. coli* strain was accompanied by a decrease in serum resistance.

Furthermore, in agreement with the cytotoxic effect of alpha-haemolysin on PMN suggested by others [31–33], our findings indicate a lower intracellular killing of alpha-haemolytic *E. coli* strains in PMN compared to non-haemolytic ones. On the other hand, survival of an other alpha-haemolytic *E. coli* strain, No. 699, in PMN was similar to that of non-haemolytic ones (Table V).

Despite the fact that the studied group included only 10 isolates, from our present and recent work [34] it may be assumed that in *E. coli* strains isolated from patients with various intestinal and/or extraintestinal infections, plasmids are frequently found and it seems to be possible that plasmids may increase resistance of bacteria to nonspecific immune mechanisms. However, further study is needed to elucidate the role of single plasmid in resistance of bacteria to bactericidal activity of serum and phagocytosis.

REFERENCES

- Kétyi, I.: Acta Microbiol Acad Sci Hung **31**, 1 (1984).
- Ørskov, I., Ørskov, F.: J Hyg Camb **95**, 551 (1985).
- Low, D.: J Med Microbiol **26**, 1 (1988).
- Timmis, K. N., Manning, P. A.: In Falmagne, P. (ed.): Bacterial Protein Toxins. Zentralbl Bakteriell (A) (Suppl) **15**, 301 (1986).
- John, J. F., Twitty, J. A.: Rev Infect Dis **8**, 693 (1986).
- Timmis, K. N., Boulnois, G. J., Bitter-Suermann, D., Cabello, F. C.: Curr Top Microbiol Immun **118**, 197 (1985).
- Siegfried, L., Puzová, H., Hlaučová, J., Ďurovičová, J., Kročková, J., Ebert, H. H.: Bratisl lek Listy **91**, 292 (1990).
- Evans, D. J. Jr., Evans, D. G., DuPont, H. L.: Infect Immun **23**, 336 (1979).
- Honda, T., Taga, S., Takeda, Y., Miwatani, T.: J Clin Microbiol **13**, 1 (1981).
- Smith, H. W.: J Pathol Bacteriol **85**, 197 (1963).
- Šmarda, J.: J Hyg Epidem (Prague) **4**, 151 (1960).
- Smith, H. W.: J Gen Microbiol **83**, 95 (1974).
- Anderson, E. S.: Nature **1016** (1965).
- Birnboim, H. C., Doly, J.: Nucleic Acids Res **7**, 1513 (1979).
- Guyer, M. S., Clark, A. J.: J Bacteriol **125**, 233 (1976).
- Miler, L., Vondráček, J., Hromádková, L.: Folia Microbiol **24**, 143 (1979).
- Benge, G. R.: J Med Microbiol **27**, 11 (1988).
- Pantazis, C. G., Kniker, W. T.: J Reticuloendoth Soc **26**, 155 (1979).
- Binns, M. M., Mayden, J., Levine, R. P.: Infect Immun **35**, 654 (1982).
- Agüero, M. E., Aron, L., DeLuca, A. G., Timmis, K. N., Cabello, F. C.: Infect Immun **46**, 740 (1984).
- Nilius, A. M., Savage, D. C.: Infect Immun **43**, 947 (1984).
- Sondell, K., Athlin, L., Björner, L., Eriksson, S., Norberg, B.: Mech Ageing Dev **51**, 55 (1990).
- Moll, A., Manning, P. A., Timmis, K. N.: Infect Immun **28**, 359 (1980).
- Agüero, M. E., Cabello, F. C.: Infect Immun **40**, 359 (1983).
- Lian, Ch., Hwang, W. S., Pai, Ch. H.: Infect Immun **55**, 1176 (1987).
- Emödy, L., Pál, T., Safonova, N. V., Kuch, B., Golutva, N. K.: Acta Microbiol Acad Sci Hung **27**, 333 (1980).
- Cavaliere, S. J., Bohach, G. A., Snyder, I. S.: Microbiol Rev **48**, 326 (1984).
- Emödy, L.: Ann Immunol Hung (Suppl I) **27**, 401 (1984).
- Hughes, C., Philips, R., Roberts, A. P.: Infect Immun **35**, 270 (1982).
- Kubens, B. S., Opferkuch, W.: Zentralbl Bakteriell (A) **270**, 52 (1988).
- Cavaliere, S. J., Snyder, I. S.: Infect Immun **37**, 966 (1982).
- Gadeberg, O. V.: Acta Path Microbiol Immunol Scand Sect B **95**, 219 (1987).
- Gadeberg, O. V., Larsen, S. O.: Acta Path Microbiol Immunol Scand Sect B **96**, 337 (1988).
- Puzová, H., Ďurovičová, J., Hocmanová, M., Kerestešová, A., Hlaučová, J., Janigová, V.: Čs Pediat **44**, 204 (1989).

THERMOPHILIC AND THERMOTOLERANT FUNGI OF ANIMALS' HAIR

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(Received October 29, 1990)

Nine thermophilic genera and 17 species in addition to one variety of *Aspergillus flavus*, *Malbranchea pulchella* and *Humicola grisea* were collected from hair samples in Riyadh, Saudi Arabia at 45 °C. Fifty-one hair specimens of rabbit, sheep, camel and horse were examined for the presence of thermophilic fungi. The most frequent species were *Aspergillus fumigatus*, *Aspergillus niger*, *Thermoascus aurantiacus* and *Malbranchea pulchella* var. *sulfurea*. In low frequency, *Aspergillus flavus*, *Aspergillus quadrilineatus*, *Paecilomyces variotii*, *Paecilomyces aeruginus*, *Mucor pusillus* and *Rhizopus stolonifer* were also recovered. The hair samples tested in the present study were completely free from any keratinolytic fungi at 45 °C.

Thermophilic fungi have been isolated from different substrates such as soils [1, 2] self-heating materials [3–6] and from birds' nests [7].

In Saudi Arabia, Ali [8], Ali et al. [9] and Fathi et al. [10] studied mesophilic fungi from soils whereas Abdel-Hafez [11] studied thermophilic fungi, but none of the studies has focused on thermophilic fungi from animals' hair. In this paper, an attempt is made to evaluate the occurrence of thermophilic and thermotolerant fungi on animals' hair in Saudi Arabia.

Materials and methods

Fifty-one hair samples were collected from different sites in Riyadh city of which 19 were from rabbits, 13 from sheep, 12 from camels and 7 from horses. These samples were placed in clean plastic bags and transferred immediately to the laboratory. The hair samples were sprinkled on sterile soil which had been placed in Petri dishes and moistened with sterile distilled water and remoistened whenever necessary and incubated at 45 °C for about three months. The moulds which appeared on the baits were transferred and cultured on plates of Sabouraud's dextrose agar with chloramphenicol. The plates were incubated at 45 °C for 3–4 weeks and fragments of the baits were examined microscopically.

Results and discussion

Nine thermophilic genera and seventeen species in addition to one variety of *Aspergillus flavus*, *Malbranchea pulchella* and *Humicola grisea* were collected from rabbit, sheep, camel and horse hair on Sabouraud's dextrose agar. The

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hair samples and the isolation plates were completely free from any keratinophilic fungi at 45 °C (Table I). The importance of temperature for the growth of keratinolytic fungi was noticed by many authors [12–15]. Pugh and Evans [14] reported that the keratinophilic fungi which were isolated from the birds were all mesophilic. Consequently, they would occur on the outer contour feathers rather than on the inner down feathers, where the temperature would be higher. The generally high body temperature of birds would also tend to preclude the occurrence of dermatophytes from those parts of the body surface which are insulated by feathers. Hubalek [16] mentioned that the body temperature of the house sparrow approximates 41 °C but within the plumage there is a marked temperature gradient ranging from about 40–41 °C (next the skin) and 34–38 °C (partly under the feathers) to 25–30 °C (outside the surface of the feathers on the under tail-coverts). Such a gradient permits the survival even of thermophobic fungi (for which exposure to 37 °C is lethal). Hubalek and Balat [15] noticed that the fungal species grow in their majority better at lower temperatures (optimum about 25 °C), whereas the temperature above 30 °C acts adversely on their growth. The minimum occurrence of keratin decomposers in summer may be explained by the interaction of higher temperature and lower humidity.

As shown in Table I, in the present study *Aspergillus* was the most frequent genus (45.1% in mammals examined). It occurred on the hair of rabbit, sheep, camel and horse in 78.9%, 23.1%, 16.6% and 42.8% of the samples, respectively. It was represented by 8 species and one variety from which *A. fumigatus* and *A. niger* were the most common. *A. fumigatus* has long been known as a thermophilic fungus, but Crisan [17] and Cooney and Emerson [3] considered it as thermotolerant as it has a maximum near 50 °C but minimum well below 20 °C. Other species of *Aspergillus* occurred less frequently.

T. aurantiacus (35.3% of all hair samples) occupied the second place. *M. pulchella* var. *sulfurea* (31.4% of mammals examined) ranked third. *Paecilomyces* (2 species) and *Talaromyces* (1 species) were isolated in low frequency and emerged in 13.7% and 11.8% of hair samples, respectively. Four genera were of rare occurrence and these were *Humicola* (3 species), *Mucor* (1 species), *Rhizopus* (1 species) and *Myriococcum* (1 species).

Besides these fungi diverse thermophilic actinomycetes and bacteria appeared in rare frequency on the hair samples incubated at 45 °C. Moustafa and Hussein [18] isolated a thermophilic actinomycete (*Thermomonospora vulgaris*) from Egyptian soil by enrichment technique, which was capable of digesting native keratin as well as autoclaved keratin (chicken feathers).

According to Ainsworth and Austwick [19] certain species of thermophilic fungi may be potential pathogens to animals including birds and man. Macdonald [20] reporting on wild bird's mortality in Britain for 1962, states that the bacterial, mycotic and viral infections caused 19% of deaths of which

Table I

The occurrence of thermophilic and thermotolerant fungi on hair samples from rabbits, sheep, camels and horses

Genera and species	Rabbit (19)	Sheep (13)	Camel (12)	Horse (7)	Total (51)	
					No.	%
<i>Aspergillus</i>	15*	3	2	3	23	45.1
<i>A. fumigatus</i> Fresenius	11	1	1	2	16	31.4
<i>A. niger</i> van Tieghem	7	1	1	2	11	21.6
<i>A. flavus</i> Link ex Fr.	3	—	—	1	4	7.8
<i>A. quadrilineatus</i> Thom and Raper	3	—	—	—	3	5.9
<i>A. ustus</i> (Bain.) Thom and Church	1	1	—	—	2	3.9
<i>A. candidus</i> Link	—	—	1	—	1	2.0
<i>A. flavus</i> var. <i>colummaris</i> Raper and Fennell	1	—	—	—	1	2.0
<i>A. terreus</i> Thom	—	—	—	1	1	2.0
<i>A. versicolor</i> (Vuill.) Tiraboschi	1	—	—	—	1	2.0
<i>Thermoascus aurantiacus</i> Miehe	11	4	1	2	18	35.3
<i>Malbranchea pulchella</i> var. <i>sulfurea</i> (Miehe) Cooney and Emerson	12	3	1	—	16	31.4
<i>Paecilomyces</i>	7	—	—	—	7	13.7
<i>P. variotii</i> Bainier	4	—	—	—	4	7.8
<i>P. aeruginus</i> Samson	3	—	—	—	3	5.9
<i>Talaromyces dupontii</i> Griffon and Maublanc	5	—	1	—	6	11.8
<i>Humicola</i>	2	—	1	—	3	5.9
<i>H. grisea</i> var. <i>thermoidae</i> Cooney and Emerson	1	—	—	—	1	2.0
<i>H. langinosa</i> (Griffon und Maublanc) Bunce	1	—	—	—	1	2.0
<i>H. insolens</i> Cooney and Emerson	—	—	1	—	1	2.0
<i>Mucor pusillus</i> Lindt	3	—	—	—	3	5.9
<i>Rhizopus stolonifer</i> (Ehrn. ex Fr.) Lindt	2	—	1	—	3	5.9
<i>Myriococcum albomyces</i> Cooney and Emerson	—	—	1	—	1	2.0
Sterile mycelium	2	—	—	—	1	2.0

* Number of isolations

the cases with aspergillosis were 4%. Tansey [21] reported that alligator nesting material provides a suitable habitat for thermophilic fungi. The heat required for growth of these fungi is presumably provided by microbial metabolism, rather than the body heat of a warm-blooded bird or mammal; sun-heating of nests should not be excluded as a possibility.

In Egypt, most of the above genera and species were isolated previously from soil at 45 °C [22, 23], from wheat and broad bean straw composts [4], from cow and sheep manure [24] and from camel dung [25]. Also they were isolated from soils in Saudi Arabia and Kuwait [11, 26].

REFERENCES

1. Emerson, R.: In Ainsworth, G. C., Sussman, A. S. (eds): The fungi. Vol III. Academic Press, New York (1968).
2. Jac, M. A., Ransey, M. R.: *Mycologia* **69**, 109 (1977).
3. Cooney, D. G., Emerson, R.: *Thermophilic fungi*, Eumycota. Freeman, San Francisco (1964).

4. Moubasher, A. H., Abdel-Hafez, S. I. I., Abdel-Fattah, H. M., Moharram, A. M.: *Mycopathologia* **78**, 169 (1982).
5. Tansey, M. R.: *Mycologia* **63**, 537 (1971).
6. Moubasher, A. H., El-Hissy, F. T., Abdel-Hafez, S. I. I., Hassan, S. K. M.: *Mycopathologia* **68**, 39 (1979).
7. Apinis, A. E., Pugh, G. J. F.: *Mycopath Mycol Appl* **33**, 1 (1966).
8. Ali, M. I.: *Bull Faculty of Science, Riyadh University* **8**, 7 (1977).
9. Ali, M. I., Abu-Zinada, A. H., El-Masharawi, Z.: *Bull Faculty of Sciences, Riyadh University* **8**, 203 (1977).
10. Fathi, S. M., El-Husseini, T. M., Abu-Zinada, A. H., *Bull Faculty of Science, Riyadh University* **7**, 17 (1975).
11. Abdel-Hafez, S. I. I.: *Mycopathologia* **80**, 15 (1981).
12. Dawson, C. O., Gentles, J. C., Brown, E. M.: *Sabouraudia* **3**, 245 (1964).
13. Dvorak, J., Hubalek, Z.: *Mycopath Mycol Appl* **38**, 305 (1969).
14. Pugh, G. J. F., Evans, M. D.: *Trans Br Mycol Soc* **54**, 241 (1970).
15. Hubalek, Z., Balat, F.: *Zentralbl Bakteriell* **131**, 179 (1976).
16. Hubalek, Z.: *Trans Br Mycol Soc* **66**, 509 (1976).
17. Crisan, E. V.: *The Isolation and Identification of Thermophilic Fungi*. M. Sc. Thesis, Purdue University Lafayette, 1959.
18. Moustafa, S. A., Hussein, A. M.: *Zentralbl Bakteriell Abt. I* **129**, 591 (1974).
19. Ainsworth, G. C., Austwick, P. K. C.: *Trans Br Mycol Soc* **38**, 369 (1955).
20. Macdonald, J. W.: *Bird Study* **12**, 181 (1965).
21. Tansey, M. R.: *Mycologia* **65**, 594 (1973).
22. Abdel-Fattah, H. M., Moubasher, A. H., Abdel-Hafez, S. I. I.: *Bull Faculty of Science, Assiut University* **6**, 225 (1977).
23. Mazen, M. B.: *Ecological Studies on Cellulose Decomposing Fungi in Egypt*. Ph. D. Thesis. Botany Department, Faculty of Science, Assiut University 1973.
24. Moharram, A. M.: *Studies on Thermophilic and Thermotolerant Fungi in Egyptian Soils*. Ph. D. Thesis, Botany Department, Faculty of Science, Assiut University (1984).
25. Moharram, A. M., Bagy, M. M. K., Abdel-Mallek, A. Y.: *Egypt J Bot* **28**, 71 (1985).
26. Moustafa, A. F., Sharkas, M. S., Kamel, S. M.: *Botany* **23**, 213 (1976).

TECHNOLOGICAL ASPECT OF THE PRODUCTION OF LIVE DYSENTERY VACCINES FOR ORAL ADMINISTRATION

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(Received October 29, 1990)

An experimental technology for the production of live freeze-dried vaccines prepared from attenuated *Shigella flexneri* 2a and *Shigella sonnei* I strains was developed. It is based on the cultivation of bacterial strains in a fermentor under conditions which ensure high yields. The strains grow in S-form, their antigenic structure is preserved and they remain non-virulent. The attenuating markers are stable. The freeze-dried vaccines retain good immunogenicity when applied intra-intestinally to rats.

Active immunoprophylaxis of shigellosis is an important but not yet solved problem. Small-scale experiments on volunteers have shown that the oral immunization of humans with live hybrid or attenuated strains is a promising approach. The oral administration of an *Escherichia coli* hybrid strain bearing surface antigens of *Shigella flexneri* ensured 50% protection after challenge with a virulent *Shigella* strain [1]. The results of the studies of the stability and immunogenicity of *Shigella sonnei* I and *S. flexneri* 2a non-virulent strains carrying two markers of attenuation and the results from preliminary tests on volunteers raised the problem of development of a technology for the large-scale production of these potential vaccinal strains [2–5]. Our experience has shown that it is possible to cultivate attenuated *S. flexneri* 2a strains in a fermentor [6, 7]. Data on the development of a technique for the production of live vaccines from *S. flexneri* 2a and *S. sonnei* I with two markers of attenuation are presented in this communication.

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Materials and methods

Bacterial strains. The construction and characterization of the attenuated strains *S. flexneri* 2a 77 $\text{ade}^- \text{rif}^r$ and *S. sonnei* I 3359 $\text{ade}^- \text{rif}^r$ has been described earlier [4, 8]. The virulent strain *S. flexneri* 2a 516 was also used in the experiments. All strains were kept in a freeze-dried state.

Cultivation conditions and their modifications. A fermentor from MBR Bioreactor, Switzerland with a working volume of 10 l was used. Cultivation experiments were performed at dissolved oxygen levels of 10, 30 or 50% at 37 °C. The pH of 7.2 was maintained automatically by the addition of a 20% sodium hydroxide solution. Cultivation lasted for 9–10 h. As nutrient medium a casein enzymatic broth with 2.2–2.4 g/l amine nitrogen produced by the Institute of Infectious and Parasitic Diseases was used. In some cultivation tests it was supplemented with yeast extract (50 g/l), obtained from Merck, FRG. The inoculum culture was incubated for 6 h with continuous shaking at 200 rpm. The ratio of the inoculum volume to the working volume of the fermentor was 1:20. The density of the bacterial suspension was determined spectrophotometrically at 650 nm. Ten optical units of the Fifth International Optical Standard for turbidity was taken to equal 10^9 cells/ml.

Freeze-drying. After the fermentor cultivation of the strains the bacterial suspensions were centrifuged and the pellet was resuspended in a cryoprotective medium containing 10–15% sucrose, 6–10% sodium glutamate and 2–3% thermally denaturated gelatin. The lyophilization process in the "Usifroid" freeze-drying apparatus lasted for 40–46 h and the final temperature of the product was 35 °C during a period of 8–10 h. The freeze-dried vaccine was kept in ampoules in vacuum or filled with an inert gas.

Characterization of the strains. The stability of the ade^- marker was controlled by plating different bacterial dilutions on M9 medium without adenine. A frequency of reversion of $\geq 10^{-7}$ was accepted as normal. The resistance of the attenuated strains to rifampicin was determined by cultivation in a medium containing 100 µg/ml rifampicin and in parallel on nutrient agar without antibiotics. Normal back mutation frequency was 10^{-7} . The antigenic structure of the strains was determined by agglutination using type- and group-specific *S. flexneri* and *S. sonnei* antisera. The strains were checked for potential S → R dissociation and their biochemical activity was regularly tested.

The degree of attenuation was assayed by the method of Serény on guinea-pig eyes [9]. The immunogenicity of the grown either on solid agar or in a fermentor before and after freeze-drying was assayed using the model of enteral immunization of rats as described previously [4]. The bacterial suspensions were injected intraintestinally and the immune response was followed from day 3 till day 7 by studying (i) the proliferation of IgM antibody producing cells in the spleens by the haemolytic plaque-forming method of Dresser and Greaves [10]; (ii) serum antibody titre and (iii) the secretion of antibodies into the gut. Antibodies in the serum and intestinal content were measured by passive haemagglutination using sheep red blood cells coated with lipopolysaccharide antigens obtained from the respective virulent strains [11]. The same coated erythrocytes were used in the plaque-forming cell method.

The vaccinal doses were quantified by comparing the density of the bacterial suspension with that of the Fifth Optical Standard and the number of viable bacteria in each dose was determined by plating on agar serial dilutions of the suspension.

Results and discussion

No significant differences in the yield of bacteria were observed in cultivation experiments in a fermentor using different dissolved oxygen levels (10, 30 or 50%) for strains *S. flexneri* 2a 77 and *S. sonnei* I 3359 (Table I). The effect of the supplementation of the nutrient medium with 50 g/l yeast extract is presented in Table II. The virulent strain grew equally well in the medium with and without yeast extract addition. However, the two attenuated strains grew better in a broth containing yeast extract — the

Table I

Cultivation of the attenuated *S. flexneri* 2a 77 and *S. sonnei* I 3359 strains in nutrient medium with 50 g/ml yeast extract in a fermentor at different dissolved oxygen levels

Strain	dO ₂ in %	Yield 10 ⁹ cm ⁻³	Maximum absolute growth rate (10 ⁹ cm ⁻³ h ⁻¹)	Maximum relative growth rate (h ⁻¹)
<i>S. flexneri</i> 2a 77	50	28	5.0	1.44
	50	28	4.8	1.22
	30	29	5.4	1.15
	10	28	6.0	1.05
	10	30	6.0	1.00
<i>S. sonnei</i> I 3359	50	28	6.0	1.25
	50	30	9.0	1.5
	30	28	14.0	1.46
	10	30	11.0	1.42

Table II

Cultivation of the attenuated *S. flexneri* 2a 77 and *S. sonnei* I 3359 strains and the virulent *S. flexneri* 2a 516 in a fermentor

Strain; number of cultivation experiments	Yeast extract added g l ⁻¹	Yield 10 ⁹ cm ⁻³	Maximum absolute growth rate 10 ⁹ cm ⁻³ h ⁻¹	Maximum relative growth rate h ⁻¹
<i>S. flexneri</i>				
2a 77	0	12.8 ± 2.4*	3.2 ± 1.0	1.05 ± 0.25
	5	28.6 ± 1.1**	5.36 ± 0.85**	1.17 ± 0.21
<i>S. flexneri</i>				
2a 516	0	31.8 ± 3.2	12.0 ± 3.3	1.08 ± 0.25
	5	33.0	17.0	1.36
<i>S. sonnei</i> I				
3359	0	12.43 ± 1.56	3.56 ± 0.3	1.05 ± 0.21
	5	27.00 ± 2.8**	8.15 ± 3.17**	1.27 ± 0.18

* Mean value ± standard deviation

** p < 0.05, Student's *t*-test

average yields and the maximum absolute growth rates were both significantly increased.

The two attenuated strains retained their markers after cultivation in a fermentor and after lyophilization. The frequency of reversion of each marker was $\geq 10^{-7}$. The strains preserved their S-form ($\geq 95\%$) and their antigenic structure. They remained nonvirulent (Table III).

Table III

Stability of the antigenic structure and of the attenuating markers of the strains before and after lyophilization

Strain	Lot No.	Viable cells (in %)		S-form of the colonies (in %)	Reversion to* rifampicin ^s adenine ⁺				KK**
		fresh culture	day 10th after freeze-drying		fresh	lyoph.	fresh	lyoph.	
<i>S. flexneri</i>									
2a 77	2, 3, 4, 5	52±16	32±11	≥98	—	—	—	—	—
<i>S. sonnei</i> I									
3359	1, 2, 3	45±15	38±5	≥96	—	—	—	—	—

* From each lot 250 colonies were tested

** KK = keratoconjunctivitis test on guinea pigs

The immunogenicity of bacterial suspensions collected from fresh agar was tested in vivo in parallel with that of a fermentor cultivated and lyophilized vaccines. The results suggested that there was no significant difference in the numbers of antibody-producing cells in the spleens of rats induced by the intraintraintestinally applied agar- or fermentor-grown bacterial cells (Table IV).

Table IV

Immunogenicity of attenuated Shigella strains — antibody producing cells in the spleens of enterally immunized rats

Strain	Cultivation conditions	Dose of bacterial cells	Viable cells in %	Number of rats	Number of APC*
<i>S. flexneri</i>	on agar	10 ¹⁰	62±12	25	40160±12018
2a 77	in fermentor	10 ¹⁰	48±18	25	36100±20126
<i>S. sonnei</i> I	on agar	10 ¹⁰	52±18	25	32520±4640
3359	in fermentor	10 ¹⁰	54±16	25	28280±6420

* Antibody-producing cells per 10⁸ lymphoid cells, peak value on day 3–5 (mean ± standard deviation)

A marked proliferation of antibody-secreting cells was also observed in the spleens of animals immunized with *S. flexneri* 2a and *S. sonnei* I freeze-dried vaccines (Table V). The antibody titres in the sera of rats treated intraintraintestinally with freshly cultivated or lyophilized vaccines were practically the same (Table VI). Furthermore, the freeze-dried *S. flexneri* 2a 77 as well as the freshly cultivated vaccine failed to induce a local secretory immune response detectable by passive haemagglutination in the intestines of rats 7 days after their application. At the same time the freeze-dried *S. sonnei* I 3359 vaccine

Table V

*Immunogenicity of attenuated Shigella strains cultivated in a fermentor before and after lyophilization**

Strain	Lot No.	Viable cells in %	Number of APC**
Before lyophilization			
<i>S. flexneri</i> 2a 77	1	52 ± 12	35467 ± 14784
	2	54 ± 10	43034 ± 20495
	3	58 ± 10	50174 ± 13650
<i>S. sonnei</i> I 3359	1	50 ± 14	48654 ± 33016
	2	54 ± 16	44420 ± 6240
	3	64 ± 18	38580 ± 12420
After lyophilization			
<i>S. flexneri</i> 2a 77	1	32 ± 13	23417 ± 16133
	2	36 ± 12	29330 ± 6150
	3	34 ± 14	29180 ± 25670
<i>S. sonnei</i> I 3359	1	38 ± 12	20450 ± 11210
	2	39 ± 14	48395 ± 3796
	3	36 ± 11	22380 ± 6400

* Groups of 25 rats were immunized enterally with 10¹⁰ bacteria

** See Table IV

induced a secretory immune response as demonstrated by the appearance of specific antibodies in the intestine (Table VI).

The technology developed based on the cultivation of attenuated *Shigella* strains in a fermentor and the subsequent lyophilization of the bacteria would permit the production of a large quantity of bacterial mass with a low cost per single vaccinal dose and a long shelf-life of the preparation. The two-marker *S. flexneri* 2a 77 and *S. sonnei* I 3359 strains produced by the method

Table VI

Antibodies in the serum and the gut content of rats immunized enterally with 10¹⁰ freshly cultivated or lyophilized Shigella strains; 25 rats per group

Strain	Lot No.	Titre of antibodies in*			
		serum		gut content	
		fresh	lyoph.	fresh	lyoph.
<i>S. flexneri</i> 2a 77	1	2860 ± 800	1980 ± 780	<10	<10
	2	2200 ± 860	1600 ± 920	<10	<10
	3	1640 ± 920	1420 ± 640	<10	<10
<i>S. sonnei</i> I 3359	1	1920 ± 590	1580 ± 420	202 ± 104	78 ± 60
	2	3440 ± 1100	1360 ± 320	220 ± 80	80 ± 40
	3	2420 ± 1400	1920 ± 860	280 ± 100	140 ± 80

* Mean value ± standard deviation on day 5 after immunization; fresh = freshly cultivated suspension used for immunization; lyoph. = lyophilized suspension used for immunization

described induced an intense and long-lasting immune response in the intestines of adult volunteers and children aged 3 to 17 years [3]. Large-scale field trials to be made will permit a final evaluation of their efficiency.

REFERENCES

1. Levine, M., Woodward, W., Formal, S., Gemski, P., DuPont, H., Hornick, R., Snyder, M.: *J Infect Dis* **136**, 577 (1977).
2. Denchev, V., Marinova, S., Linde, K., Keller, H., Trifonova, A.: *Proc 5th National Congress of Microbiology*. Varna, Bulgaria 1981. Part 1, pp. 95-99.
3. Denchev, V., Marinova, S., Vassilev, T., Bratoeva, M., Linde, K.: *Vaccine* **8**, 30 (1990).
4. Denchev, V., Summers, T., Marinova, S., Vassilev, T., Panova, I., Linde, K.: *Acta Microbiol Hung* **37**, (1990).
5. Marinova, S.: D. Sc. Thesis, Sofia 1987.
6. Nenkov, P., Bratoeva, M., Vitanov, T., Marinova, S., Denchev, V., Linde, K.: *Vaccine* **5**, 25 (1987).
7. Nenkov, P., Bratoeva, M., Vitanov, T., Marinova, S., Denchev, V., Linde, K.: *Zh Microbiol Epidemiol Immunobiol* **5**, 6 (1988).
8. Linde, K., Denchev, V., Bondarenko, V., Marinova, S., Ranghagen, B., Bratoeva, M., Tsvetanov, Y., Romanova, Y.: *Vaccine* **8**, 25 (1990).
9. Serény, B.: *Acta Microbiol Acad Sci Hung* **4**, 367 (1957).
10. Dresser, D., Greaves, N.: In Weir, D. (ed.): *Handbook of Experimental Immunology*. Blackwell, Oxford 1972. p. 1.
11. Thilo, W., Krieclin, J.: *Gesundheitswesen* **27**, 1268 (1969).

INVESTIGATIONS INTO VIRUS CARRIERSHIP IN HUMAN SEMEN AND MOUSE TESTICULAR CELLS

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(Received November 7, 1990)

Virological investigations were performed to approach the mechanism of male infertility and recurrent abortions. Infectious adenovirus or herpes simplex virus was found in nearly 40% of semen samples obtained from sterile men. The viruses were detected in latent form in 60% of the cells. The same viruses as in the father's cells were found in the aborted material. In vitro inoculation of cell cultures with material of semen origin made the presence of other latent viruses probable. The affinity of adeno- and herpesviruses to urogenital organs was supported by animal experiments in which a new method of in vivo intratesticular inoculation was applied. The close interaction between urogenital cells and viruses was also supported by in vitro infection of human semen with adeno- or herpesviruses. The viruses absorbed to cells, penetrated the latter and their components replicated inside. It is suggested that local chronic viral infection may play part in male infertility and some cases of the recurrent abortions through damaging of cells. In patients treated with Zovirax, virus carriership ceased and the normal function of sperms started again.

The etiology of some cases of sterility and chronic prostatitis of men is still unclear. It has been claimed that semen samples may carry cytomegalovirus (CMV), hepatitis B virus (HBV) or human immunodeficiency virus (HIV) [1–3.] In animal experiments even long-lasting persistence of CMV has been reported in sperms and testicular cells [1, 4]. Relied upon previous data, we examined sperms and testicular cells of infertile men and of husbands whose wives had repeatedly aborted. We wanted to show whether such cells may carry adenovirus or HSV of high affinity to urogenital organs. In the present work, we tried to support our observations in men by infecting the testis of mice in situ. We developed a new method for intratesticular inoculation of mice.

Patients and methods

Patients. Semen samples were examined from 73 men. Of these, 39 had presented at andrological consultation because of sterility; in 10 cases the wife had aborted repeatedly; semen samples from 9 healthy men were examined in vitro with viruses added by us; those from 15 healthy men served as control. Aborted materials were examined in 5 cases.

Experimental animals. Male CFLP mice 8–10 weeks old were used.

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Viruses. International identified adenovirus type 2, 12, 19 and herpes simplex virus type 1, 2 were applied.

Virus infection. Thirty male mice were intraperitoneally infected with adenovirus or HSV, then 1, 3 and 7 days later attempts were made to demonstrate localization of the viruses in the urogenital organs. Further 30 mice were inoculated with the same viruses directly into the testis, unilaterally. For this purpose we developed a new method, which in contrast with the techniques reported so far, does not require opening of the abdominal cavity. By palpation the testis could be found, fixed by fingers and inoculated in situ with viruses or Indian ink. The rate of error of the method was very low. The contralateral testis remained intact and served as control. As controls further 20 mice of the same weight and age were used. Some of these were not inoculated at all, others were given intratesticularly saline instead of virus. The animals inoculated by HSV and adenovirus (mainly adenovirus type 19 — oculogenital type) were killed one day and three days after inoculation, respectively. Their abdominal wall was incised and the testes were lifted out to be examined.

Indian ink inoculation. To demonstrate as to whether it is possible to infect the testis directly without opening the abdomen cavity, and leaving the contralateral testis intact, we injected Indian ink into one of the testes and observed whether the dye appeared in the contralateral testis. Animals were killed one day or three days after inoculation.

Virus (re)isolation and identification of virus antigens. Attempts were made in cell cultures to (re)isolate virus from both human semen and testicular samples from mice. As primary cultures, human amnion and human embryonic fibroblast cultures were employed. In addition, the continuous cell line HEp-2 was used. An aliquot of 0.1 ml fresh semen sample or the supernatant of a suspension of disintegrated mouse cells was added to cell cultures. After co-incubation, cytopathic changes were observed under the light microscope.

The samples, both of human and mouse origin, were examined for viral antigens by a method of Coons and Kaplan as modified by us, in a Zeiss Fluoval microscope at magnification $\times 600$ [5]. The isolates were identified by virus-neutralization tests.

Virus antisera. Hyperimmune viral antisera used in immunofluorescence studies and in virus neutralization tests were prepared against adenovirus type 2, 12, 19; HSV-1, 2 and CMV.

Results

As an effect of inoculation of cell cultures with some samples of human semen, changes suggestive of a cytopathic agent appeared several days post-inoculation. Since even the human semen is slightly toxic for cultured human cells, the supposed agent was subjected to consecutive passages in cell cultures. Thus, toxicity was eliminated and the cytopathic changes became well-visible. After passages, 38% of the 64 samples examined because of male sterility or recurrent abortion of wife, and 20% of the control samples yielded a cytopathic agent, viz. virus. The isolates were identified by virus-neutralization tests. Latent and oncogenic viruses (adenovirus, HSV or CMV) were detected at about the same frequency in samples from sterile men. All samples from cases with recurrent abortions, except one, yielded one, sometimes two, of the above-mentioned viruses. The cells of miscarried fetuses and their membranes always carried the same virus(es) as the husband's semen.

From a low percentage of the control men's semen samples HSV, but no other viruses could be recovered, although, in a few cases cell cultures incubated with the control sample developed cytopathic changes reminding of the characteristics of papovavirus, rubella virus, measles virus, varicella-zoster virus or some other viral agent.

Virus antigens (indicating carriership) were looked for by the IF test. Latent carriership of adeno- and/or herpesvirus antigen was detected in 60% or more, i.e. more frequently than carriership of infectious virus. All cells of the miscarried fetuses and placentas contained virus.

Figure 1 shows sperms carrying and not carrying virus. The former being much higher in number, showed a greenish autofluorescence, in contrast with the latter, the heads of which fluoresced in a bright yellow colour indicating specific viral protein(s).



Fig. 1. Sperms showing in majority autofluorescence. Other sperms carry viral proteins binding to fluoresceine isothiocyanate-conjugated viral antibodies

Semen samples from control men carried viral antigen(s) but very rarely. The healthy human sperms that had been incubated with adeno- or herpesvirus showed the following in the *in vitro* immunofluorescence test: an incubation for five hours or one day was followed by a reaction between sperms or testicular cells and antigens of various types of adeno or herpesvirus. As a result, in case of herpesvirus antigens a bright fluorescence was detected in the heads of sperms and in testicular cells. Since a great part of the sperms were disintegrated, fluorescence was observable in not more than 10% of the cells. Of the adenovirus types, those tending to persist in the latent condition were adsorbed to the cells poorly, whereas the oncogenic types and the so-called

oculogenital type (type 19) showed a more intense reaction not only with the heads, but along the whole sperms. The binding of adenoviruses to testicular cells was less intense. In contrast with HSV, adenoviruses did not damage sperms.

We failed to evaluate the localization of viruses in the genital organs of animals inoculated intraperitoneally. After local infection of the testis, the following changes were observed with the IF method: both the HSV and the adenoviruses absorbed to the testicular cells and also to sperms. Part of the

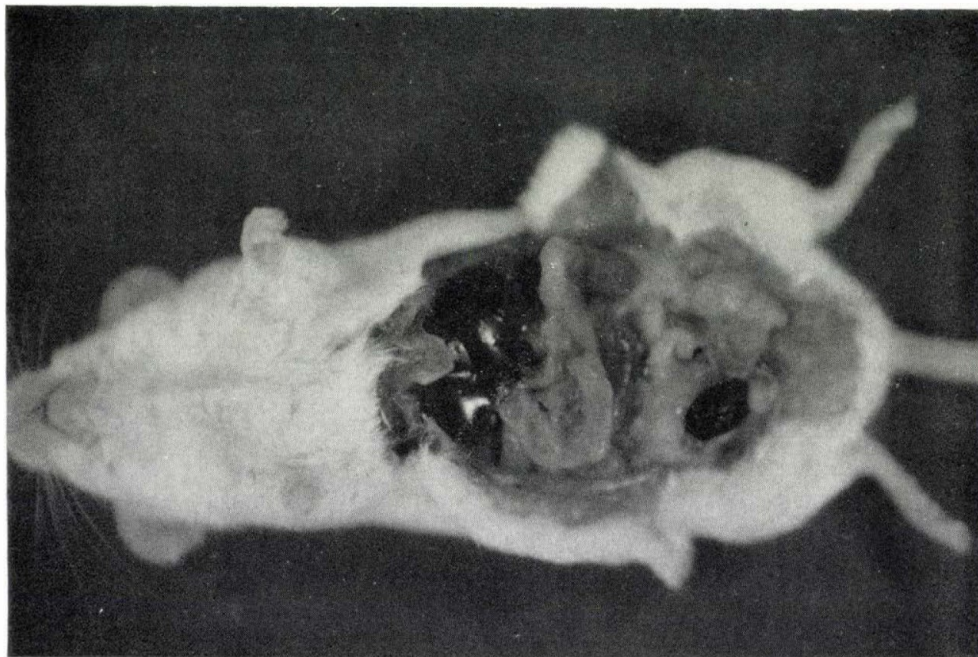


Fig. 2. A mouse inoculated with Indian ink a few days before sacrificing. The inoculated (right) testis is full of black granules, all the other organs, including the left testis, contain no Indian ink

antigen(s) penetrated the cells, and some viral protein components showed replication. The negative results with control cells and the absence of detectable viral antigen in contralateral cells are in agreement with a specific replication. That the unilateral infection of the testis remained localized was supported by the unilateral intra-testicular inoculation with Indian ink (Fig. 2): the testis inoculated with this dye before the animal was killed was full of dye granules while similar granules did not appear elsewhere, they being absent in the contralateral testis.

When we made attempts to culture virus from animals' testicular cells carrying latent virus, we failed to re-isolate infectious virus. It is therefore

probable that local genital infection with adenovirus or HSV does not tend to generalize, though, these viruses are capable of replicating locally, of showing replication of antigen and of bringing about latent infection. Persistence of virus in genital cells of mice was still detectable several weeks after infection.

Discussion

The pathomechanism of some virus infections has not been satisfactorily clarified yet. It is well-known that if HSV penetrates the organism through the damaged skin or mucous membrane — in either the oral or genital region — the manifest or latent primary infection often is followed by a relapse. The relapse can be explained as follows: the virus having replicated in epithelial cells at the site of penetration travels along sensory nerves and thus reaches the corresponding nerve cells. There, the virus can persist, sometimes even life-long. Due to environmental factors or a change in the internal milieu, the latent virus may be activated and travelling centrifugally, may reach the site of its penetration; replicating there, it may cause characteristic vesicular changes. It is also known that the fetus or newborn, if gets infected with HSV, often develops herpetic septicaemia with severe consequences [6].

On the other hand, the fate of the herpesvirus is unknown if being carried in the semen or vaginal discharges, reaches the partner's genitals. According to our experiments with human materials and results of our animal experiments, the virus can cause in such cases local latent infection in the partner's genital organs. The consequence may be prostatitis, orchitis, epididymitis, vaginitis, metritis, adnexitis or other inflammation. We have not seen generalization of the inoculated virus; the infection localized unilaterally. The individuals infected latently may become source of infection for the partner. If the viruses attack sperms and/or testicular cells, male infertility may be a further consequence of infection. Infertility may develop so that primary damaging of testicular cells makes spermatogenesis insufficient: the newly-formed spermatozoa will be few in number and poor in function, as it has been proved in animal experiments with CMV [1].

The role of adenoviruses in infections of urogenital organs is still less known, for these viruses do not cause easily observable changes in the skin and mucous membranes. According to literary data, one of the adenovirus types often attacks the eyes and at the same time, the urogenital organs [7]. Adenovirus has already been found in vaginal discharges as well, and one of its human types is considered to be the causative agent of haemorrhagic cystitis [8, 9]. It should be noted, furthermore, adenovirus has already been isolated from urine samples of AIDS patients [10].

According to our earlier studies, 6–8% of clinically symptomless and colposcopically negative women carried adenovirus in their cervix cells and

the rate of carriership was almost the same with latent HSV [6]. Symptomless carriership of viruses in the genital organs is of interest from a further respect: viruses are potentially oncogenic [6, 11, 12].

The affinity of adeno- and herpesviruses to the urogenital organs and the participation of these viruses in pathological processes is supported by our results indicating that human semen can be infected with these viruses in vitro and that the infection remains in sperms and testicular cells latent. Since the frequency of virus infections is steadily increasing, because of frequent partner change among others, it is not surprising that the presence and some role of viruses should be reckoned with in the fields of both diagnostics and therapy. In this respect, our results indicating that viral infectedness may be a factor leading to male sterility and recurrent abortions are of interest. It is another interesting observation of ours that when carriers of latent DNA viruses were given an antiviral Zovirax cure or were treated with the immunostimulant Isoprinosin, their semen ceased carrying virus and its function became correct again. Possibly, stopping of long-lasting carriership may protect even against further development of some malignancies.

It may also be of importance that, in cell cultures, phenomena suggestive of the presence of viruses other than adeno- and herpesviruses appeared on the effect of sperms being under testing for urogenital virus carriership and that recently an increasing evidence suggests that a considerable number of viruses being in a close interaction with one another co-operate in the development of malignancies [12, 13].

REFERENCES

1. Basker, J. F., Stanat, S. C., Eng-Shang Huang: *Infect Immun* **40**, 726 (1983).
2. Hollós, I., Kulcsár, G., Ongrádi, J., Nász, I., László, B.: Hepatitis viruses. Viral hepatitis. (Hung.) Medicina, Budapest 1986.
3. Horváth A.: AIDS. Acquired Immunodeficiency Syndrome (Hung.). Medicina, Budapest, 1985.
4. Lang, D. J., Kummer, J. F.: *J Infect Dis* **132**, 472 (1975).
5. Dán, P., Geck, P., Kulcsár, G., Nász, I.: *Acta Microbiol Acad Sci Hung* **19**, 51 (1972).
6. Kulcsár, G., Nász, I., Dömötöri, J., Dán, P., Horváth, J.: *Orvostudomány aktuális problémái* **41**, 53 (1981).
7. Harnett, G. B., Newnham, W. A.: *Br J Vener Dis* **57**, 55 (1981).
8. Lavery, C. R., Russel, P., Black, J., Kappagoda, N., Benn, V., Booth, N.: *Acta Cytol* **21**, 114 (1977).
9. Numazaki, Y., Shigeta, S., Kumasaka, T.: *N Engl J Med* **278**, 700 (1968).
10. De Jong, P. J., Valderrama, G., Spigland, I., Horwitz, M. S.: *Lancet* **1**, 1293 (1983).
11. Bernard, R., Schrier, P. I., Houweling, A., Boss, J. L., van der Eb, A. J., Zijlstra, M., Malief, J. M.: *Nature* **305**, 776 (1983).
12. Everett, R. D., Dunlop, M.: *Nucleic Acids Res* **12**, 5969 (1984).
13. Kulcsár, G.: Persisting adeno- and herpes simplex viruses in human pathology. Thesis. (Hung.) Budapest 1975.

TEMPERATURE INDUCED CHANGES IN MICROSOMAL (Na⁺, K⁺)-ATPase AND LIPID COMPOSITION OF *CANDIDA KEFYR*

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(Received September 13, 1990)

Growth temperature affected both the membrane lipid composition and microsoma (Na⁺, K⁺)-ATPase activity of *Candida kefyri*. Higher growth, temperature (37 °C) increased the amount of total lipids, phospholipids and free sterol. Ratios of phosphatidylcholine to phosphatidylethanolamine as well saturated to unsaturated fatty acids increased with a rise in growth temperature. K_m of the ATPase isolated from the yeast grown at 27 °C was minimum, suggesting that the membranes of *C. kefyri* grown at optimal growth temperature provide the most suitable environment for the activity of ATPase.

Alterations in growth temperature of the organism induce various changes in membrane lipid composition [1–3]. Membrane fluidity, which depends upon its lipid composition, controls various membrane functions such as permeability and transport and activity of membrane bound enzymes [4, 5]. The involvement of polar lipids in the functioning of ATPase has been demonstrated with preparations from animal tissues and microorganisms [6, 7]. (Na⁺, K⁺)-ATPase is one of such membrane bound enzymes which has an important role in ion transport [8–10]. The present investigation was carried out to understand whether changed membrane lipid composition at different growth temperatures affects the activity of (Na⁺, K⁺)-ATPase in the yeast *Candida kefyri*.

Materials and methods

Chemicals. Adenosine-5- α -triphosphoric acid (ATP, disodium salt), histidine, digitonin and α -bipyridyl were purchased from M/s Sigma Chemical Co., USA. All other chemicals were of analytical grade and were procured from local commercial sources.

Growth of yeast cells. *C. kefyri* cells were grown in 500 ml Erlenmeyer flasks containing 100 ml of the YEPG medium. The composition of the growth medium per litre was: glucose, 10 g; peptone, 5 g; yeast extract, 2.5 g and malt extract, 2.5 g. Before inoculation, the medium was autoclaved at 15 lb pressure for 20 min and cooled to room temperature. The yeast cells were grown at 20, 27, 32 and 37 °C. Log phase cells were harvested by centrifugation at 12 000 g for 20 min after 24 h. The pellet was washed once with distilled water and twice with 0.2 M Tris-HCL buffer, pH 6.7, to drain off adhering metabolites and unused ingredients of the medium.

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Preparation of microsomal fraction. Microsomal fraction was prepared as described by Hodges and Leonard [11]. The yeast cells were homogenized at 4 °C in 0.2 M Tris-HCl buffer, pH 6.7, containing 0.25 M sucrose, 1 mM EDTA and 5 mM mercaptoethanol. The ratio of cells to buffer used was 1:5 (w/v). The homogenate was centrifuged at 15 000 g for 20 min. The supernatant was further centrifuged at 100 000 g for 60 min to get microsomal fraction. The fraction was washed twice with the same buffer. The washed fraction was resuspended in 0.2 M Tris-HCl buffer at a concentration of 5 mg protein/ml and stored at -20 °C until used.

Assay of (Na⁺, K⁺)-ATPase. The method of Lundberg et al. [12] was used and 2.2 ml of the assay mixture contained 40 mM histidine buffer pH 6.7, 0.55 mM MgCl₂, 150 mM NaCl, 5 mM KCl and 4.4 mM ATP as substrate. The enzyme was preincubated with the assay mixture except substrate at 30 °C for 5 min. The reaction was started by adding the substrate. After 30 min of incubation at 30 °C, it was stopped by adding 0.1 ml of 10% trichloroacetic acid. The denatured proteins were removed by centrifugation and the phosphorus content of the clear supernatant was estimated by the method of Ames [13]. The activity of ATPase was expressed as μ moles of phosphorus released/mg protein/30 min. The protein content of the enzyme preparation was estimated by the method of Lowry et al. [14].

Extraction of total lipids and their analyses. The method of Folch et al. [15] was used for the extraction and purification of total lipids. Phospholipids were separated by thin layer chromatography. Silica gel G plates were developed in a solvent system chloroform:methanol:water 65:25:4 (v/v). In this system, partial overlapping of phosphatidylinositol and phosphatidylserine was observed which were then separated by rechromatography using the solvent system chloroform:methanol:ammonia 65:35:5 (v/v). Various spots were visualized by using iodine and identified by comparing their R_f values with authentic standards and using specific spray reagents. Total and individual phospholipids were assayed as described by Hossack and Rose [16] with slight modifications. Phospholipids were eluted from silica gel with two portions 3 ml of chloroform:methanol:water 5:5:1, (v/v/v), followed by 3 ml of methanol:acetic acid:water 95:1:5, (v/v/v). Phospholipids were determined by assaying the phosphorus content of extract using the method of Ames [13]. Total and free sterols were determined by the method of Sperry and Webb [17]. α -Tocopherol was estimated by the method of Gaunt and Barlow [18]. Data were analyzed statistically using Student's *t* test.

Results and discussion

The activity of microsomal (Na⁺, K⁺)-ATPase from *C. kefyri* cells grown at different temperatures was studied and the results are shown in Fig. 1. As the growth temperature was increased from 20 to 27 °C, the activity of (Na⁺, K⁺)-ATPase increased from 3.35 to 4.40 μ g of phosphorus released/mg protein/30 min. Further increase of growth temperature decreased the activity of the enzyme. It is noteworthy that the optimal growth temperature of *C. kefyri* is 27 °C and the activity of the ATPase isolated from the organism grown at this temperature was maximum. This indicated that the membranes of *C. kefyri* grown at optimal growth temperature provide the best environment for the activity of ATPase. The activity of membrane bound enzymes alters with the change in lipid composition of the membranes [5].

In order to examine if the changes in ATPase activity of the yeast with growth temperature can be correlated with the changes in membrane lipid composition, the effect of growth temperature on lipid composition of *C. kefyri* was studied (Table I). As the growth temperature of *C. kefyri* was increased from 20 °C to its optimal i.e. 27 °C, the lipid content increased from 0.92 to 2.70% of the dry weight ($p < 0.05$) while further rise in growth temperature to 37 °C decreased the total lipid content slightly i.e. 2.44% ($p < 0.05$) (Table

Table I

Effect of growth temperature on lipid composition of C. kefyr

Growth temperature (°C)	Total lipids (% dry weight)	Phospholipids (% total lipids)	Phospholipids (relative percentage)						Free sterols (% total lipids)	Free sterol to phospholipid (molar ratio)	α -tocopherol content
			pE	pC	pI	PS	LpH	pC:pE			
20	0.92	7.00	27.25	25.62	15.82	25.91	5.40	0.94	2.00	0.56	1.50
	± 0.09	± 0.64	± 0.53	± 0.61	± 1.46	± 0.94	± 0.56		± 0.02	± 0.02	± 0.16
23	1.82	10.50	28.92	27.23	17.79	22.85	3.20	0.94	2.60	0.48	0.93
	± 0.13	± 1.46	± 0.36	± 0.49	± 0.66	± 0.31	± 0.31		± 0.06	± 0.03	± 0.09
27	2.70	12.40	29.68	28.77	18.25	21.86	3.22	0.97	3.00	0.47	0.81
	± 0.19	± 1.54	± 0.64	± 0.43	± 1.01	± 0.34	± 0.32		± 0.09	± 0.04	± 0.07
32	2.51	14.00	25.29	31.62	17.28	21.69	3.15	1.25	3.75	0.52	0.79
	± 0.14	± 0.88	± 1.08	± 0.79	± 0.53	± 0.54	± 0.29		± 0.15	± 0.05	± 0.06
37	2.44	14.50	23.98	36.58	16.85	20.85	1.74	1.53	4.00	0.54	0.74
	± 0.11	± 1.18	± 1.03	± 0.39	± 0.53	± 0.36	± 0.13		± 0.31	± 0.06	± 0.05

pE = phosphatidylethanolamine; pC = phosphatidylcholine; pI = phosphatidylinositol; LpH = lysophospholipids

I). Ray et al. [20] also reported an increase in the lipid content of *Thermus aquaticus* with rise in growth temperature but the opposite is true for *Candida lipolytica* [1]. Phospholipids of *C. kefyri* increased steadily with rise in growth temperature. Along with the phospholipid content, free sterol also increased from 2.0 to 4.0% ($p < 0.01$) as the growth temperature was raised from 20 to 37 °C. Although both phospholipid and sterol contents increased with a rise in growth temperature, sterol to phospholipid ratio did not remain constant. It was minimum at 27 °C i.e. optimal growth temperature. The changes in free sterol to phospholipid ratio might be a result of difference in their rate of

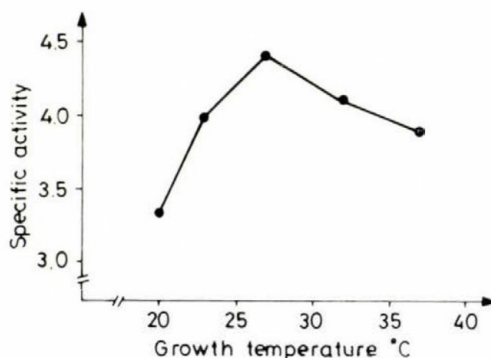


Fig. 1. Effect of growth temperature on microsomal (Na^+ , K^+)-ATPase activity of *C. kefyri*. Specific activity: μg of phosphorus released/mg protein/30 min

increase with the growth temperature. Sterols act as a regulator of acyl chain packing. Above the phase transition temperature they reduce the mobility of the acyl chain while below the transition temperature, the presence of cholesterol increases the mobility [21, 22]. The decrease in the activity of (Na^+ , K^+)-ATPase with increased level of sterol further supports the results of Yeagle et al. [23]. So it can be suggested that the increase in relative sterol content with deviation from the optimal temperature might help the *C. kefyri* cell membranes in their homeoviscous adaptation which is very important for the functioning of membrane proteins.

It was observed that the content of α -tocopherol decreased from 1.50 to 0.74 mg/g lipids ($p < 0.01$), as the growth temperature increased from 20 to 37 °C. A similar decrease of α -tocopherol in *Candida humicola* has also been reported [24]. Phospholipids are susceptible to oxidation and α -tocopherol being an antioxidizing agent might be important. Secondly, α -tocopherol is an integral part of cell membrane and has been reported to affect the permeability

properties of artificial membranes and these properties perhaps result from a structural relationship between isoprenic units of α -tocopherol and the polyunsaturated fatty acid residues of membrane lipids [25, 26]. So α -tocopherol content, which varies with the growth temperature, might be inducing some changes in the structure of cell membranes and thus might be affecting the activity of membrane bound (Na^+ , K^+)-ATPase.

Growth temperature also affected the composition of phospholipids (Table I). The phosphatidylethanolamine content was maximum at 27 °C and decreased slightly with a shift in growth temperature to either side ($p < 0.01$). However, phosphatidylcholine increased steadily with rise in growth temperature. These results are supported by the similar changes in case of *Microsporum gypseum* and *M. cookei* [2]. Studies with *Neurospora crassa* also showed that phosphatidylethanolamine increased but phosphatidylcholine decreased with a decrease in growth temperature [27]. The ratio of phosphatidylcholine to phosphatidylethanolamine, which is an important factor controlling the physical state of membranes, decreased (1.53 to 0.94) on lowering the growth temperature of *C. kefyr* from 37 °C to 20 °C.

Fatty acid composition of *C. kefyr* cells responded to the variation in growth temperature (Table II). Increase in growth temperature raised the palmitic acid content from 18.65% at 20 °C to 21.98% at 32 °C while $\text{C}_{16:2}$ showed a decrease in its content. A marked decrease was observed in $\text{C}_{18:3}$ (10.98% to 5.04%) on increasing the temperature from 20 to 37 °C. No definite trend was observed for the rest of the individual fatty acids. However, the ratio of saturated to unsaturated fatty acids increased on increasing the growth temperature. Such alterations affect the physical state of the membrane. Large portion of saturated fatty acids tend to decrease the membrane fluidity.

All these studies show that quantitatively marked changes occur in the lipid composition of *C. kefyr* with growth temperature and those changes might be responsible for the variation in (Na^+ , K^+)-ATPase activity of yeast cells when growth at different temperatures.

The activity of *C. kefyr* (Na^+ , K^+)-ATPase depends upon its growth temperature (Fig. 1). In order to know whether the change in specific activity with growth temperature is also accompanied with changes in its properties, the effect of growth temperature on K_m and V_{max} , determined from the direct linear plots of Eisenthal and Carnish-Bowden [28], was studied (Table III). The K_m of the enzyme isolated from the cells grown at 27 °C which happens to be its optimal growth temperature, was minimum and increased as the growth temperature was shifted to either side. This indicated that ATPase located in the cellular membranes of the yeast grown at optimal growth temperature has maximum affinity for the substrate ATP and hence the maximum activity. As discussed earlier, the lipid composition of *C. kefyr* cells varied as the growth temperature was changed. The change in K_m and the

Table II

Effect of growth temperature on the fatty acid composition of C. kefir

Growth temperature (°C)	Fatty acids (relative percentage)													S:US
	12:0	14:0	14:1	16:0	16:1	16:2	18:0	18:1	18:2	18:3	22:0	22:1	22:2	
20	0.60	1.50	—	16.65	10.22	2.84	6.00	32.46	15.22	10.98	0.38	0.88	0.71	0.33
23	0.97	1.91	0.15	20.29	6.94	1.79	3.20	33.34	18.00	10.54	—	0.27	—	0.38
27	1.50	4.07	—	21.12	7.93	—	3.98	34.85	14.84	8.52	0.58	—	—	0.44
32	1.92	2.80	0.51	21.98	6.30	0.56	4.02	32.27	18.16	5.58	0.38	2.08	0.76	0.46
37	1.77	2.76	—	20.73	9.65	0.81	6.47	30.33	16.83	5.04	0.97	1.03	1.47	0.48

Not detected; S = saturated fatty acids; US = unsaturated fatty acids. Results are mean of six independent experiments

activity of the ATPase might be the result of the changes in lipid composition of the yeast membrane with changed growth temperature.

The optimum pH for the enzyme in Tris-HCl buffer was found to be 7.5 and incubation temperature as 40 °C and were not affected by growth temperature. Temperature profiles of the (Na⁺, K⁺)-ATPase were also not affected by the changes in growth temperature.

Table III

*Effect of growth temperature on K_m and V_{max} values for *C. kefir* microsomal (Na⁺, K⁺)-ATPase*

Growth temperature (°C)	K_m (mM)	V_{max} (μ g of phosphorus released/mg protein/30 min)
20	3.40	5.65
23	3.30	6.80
27	3.15	7.25
32	3.35	5.75
37	3.50	7.15

The present studies showed that the growth temperature of *C. kefir* cells affects the affinity of microsomal (Na⁺, K⁺)-ATPase for its substrate ATP (K_m) and hence its specific activity. This change in K_m might be a result of the changes in lipid composition of the yeast cell membrane with growth temperature.

REFERENCES

1. Kates, M., Baxter, R. M.: In Rose, A. H., Harrison, J. (eds): The Yeast. Vol. 2. Academic Press, New York 1962. pp. 228.
2. Bansal, V. S., Khullar, G. K.: Ind J Biochem Biophys **18**, 74 (1981).
3. Durairaj, G., Vijay Kumar, I.: Biochim Biophys Acta **770**, 7 (1984).
4. Ginsberg, B. H., Jabour, J., Spector, A. A.: Biochim Biophys Acta **690**, 157 (1982).
5. Sanderman, H. Jr.: Biochim Biophys Acta **515**, 209 (1978).
6. Hodges, T. K.: In Luttge, U., Pitman, M. G. (eds): Encyclopedia of Plant Physiology, Transports in Plants IIA. Vol. 2. Springer-Verlag, Heidelberg 1976. p. 260.
7. Marre, E.: Ann Rev Plant Physiol **30**, 273 (1979).
8. Poole, R. J.: Ann Rev Plant Physiol **29**, 437 (1978).
9. Dahl, J. L., Hokin, L. E.: Ann Rev Biochem **43**, 327 (1974).
10. Racker, E.: In Biebisch, G., Tosteson, D. C., Ussing, H. H. (eds): Membrane Transport in Biology. Vol. 1. Springer-Verlag, Heidelberg 1978. p. 259.
11. Hodges, T. K., Leonard, A. T.: Methods Enzymol **32**, 392 (1974).
12. Lundberg, T., Widell, S., Larsson, C.: Physiol Plant **52**, 89 (1981).
13. Ames, B. M.: In Neufold, C. F., Ginsburg, V. (eds): Methods in Enzymology. Vol. 3. Academic Press, New York 1960. p. 169.
14. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J.: J Biol Chem **193**, 265 (1951).
15. Folch, J. M., Lees, M., Stanley, G. H. S.: Biol Chem **266**, 497 (1951).
16. Hossack, J. A., Rose, A. H.: J Bacteriol **127**, 67 (1951).
17. Sperry, W., Webb, M.: J Biol Chem **187**, 97 (1950).

18. Gaunt, J. K., Barlow, S. M.: In Macormick, D. B., Writht, L. D. (eds): *Methods in Enzymology*. Vol. 18C. Academic Press, New York 1971. p. 399.
19. Luddy, F. E., Barford, R. A., Herb, S. F., Paul, M.: *J Amer Oil Chem Soc* **45**, 549 (1968).
20. Ray, P. H., White, D. C., Brock, T. D.: *J Bacteriol* **108**, 227 (1971).
21. Oldfield, E., Keough, K. M., Chapman, D.: *FEBS Lett* **20**, 334 (1972).
22. Cogen, U., Shinizky, M., Mebec, M., Nishida, T.: *Biochim Biophys Acta* **12**, 442 (1973).
23. Yeagle, P. L., Young, J., Rice, D.: *Biochemistry* **27**, 6449 (1988).
24. Saggu, K.: M. Phil Thesis. Guru Nanak Dev University, Amritsar 1985.
25. Fukuzawa, K., Ikeno, H., Tokumura, A., Tsukatani, H.: *Chem Phys Lipids* **23**, 13 (1979).
26. Lucy, J. A.: In de Duve, D., Hayaishi, O. (eds): *Tocopherol, Oxygen and Biomembranes*. Elsevier/North-Holland, Amsterdam 1978. p. 109.
27. Martin, C. E., Siegel, D., Aaronson, L. R.: *Biochim Biophys Acta* **665**, 399 (1981).
28. Eienthal, R., Carnish-Bowden, A.: *Biochem J* **139**, 715 (1974).

MODEL EXAMINATION OF SELECTIVE MEDIA FOR ISOLATION OF *LISTERIA* STRAINS

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(Received December 17, 1990)

During the Tenth International Symposium on Listeriosis (Pécs, Hungary, 1988) the Working Party on Culture Media of IUMS-ICFMH suggested comparative examination of nine enrichment broths and nine solid selective media. On the basis of this proposal the following media were studied: LiCl–phenylethanol–moxalactam agar (LPM), polymyxin–acriflavine–LiCl–ceftazidime–aesculin–mannitol agar (PALCAM) No. 1 (home made) and No. 2 (Merck), acriflavine–ceftazidime agar (AC), Oxford agar, tripaflavine–nalidixic acid serum agar (TNSA) and Forray's agar. The study was performed as described in "Testing methods for use in quality assurance of culture media". Oxford agar proved to be the best medium. LPM, AC and Forray's agars were somewhat more inhibitory than Oxford medium. In productivity TNSA and PALCAM media were weakest but the latter one was more selective. When 43 sausage samples were enriched in UVM broths and subcultured on the above mentioned media the number of positive samples was the same on Oxford, LPM, AC and TNSA agars but it was lower on PALCAM agar No. 1. When 103 milk samples were subcultured on TNSA and PALCAM agar No. 2, the number of positive samples was the same.

Serious outbreaks of human listeriosis and the increasing number of single cases have induced a new wave of research on listeriosis [1, 2]. In consequence of these events new methods were published [3–5]. There were Methodological Round Table Discussions and a Working Party Meeting of IUMS-ICFMH at Pécs in 1988 in the frame of the Tenth International Symposium on Listeriosis [6]. During the meeting as one of the most important tasks the selection of the best media and methods for practice was mentioned. Comparative examination of enrichment broths and solid selective media was suggested. This type of studies have been coordinated by both the IUMS-ICFMH Working Party on Culture Media and the IDE/ISO/AOAC. On the basis of this proposal we compared the following media: LiCl–phenylethanol–moxalactam agar (LPM), polymyxin–acriflavine–LiCl–ceftazidime–aesculin–mannitol agar (PALCAM), acriflavine–ceftazidime agar (AC), Oxford agar, tripaflavine–nalidixic acid serum agar (TNSA) and Forray's agar. Blood agar was used as reference medium.

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Materials and methods

Media. The following media were used. Unless otherwise indicated, ingredients are given in grams.

Nutrient broth and blood agar were prepared as usual [7].

Lithium chloride-phenylethanol-moxalactam (LPM) agar [8]. Pancreatic digest of casein, 5.0; peptone (peptic digest of animal tissue), 5.0; beef extract, 3.0; sodium chloride, 5.0; lithium chloride, 5.0; glycine anhydride (Sigma G-7251), 10.0; 2-phenylethanol (Sigma P-6134), 2.5; sodium or ammonium moxalactam, 0.02; agar, 15.0; distilled or deionized water, 1000.0; Incubation lasted for 20–40 h at 30 °C.

Polymyxin-acriflavine-lithium chloride-ceftazidime-aesculin-mannitol (PALCAM) agar [9]. Columbia agar, 39.0; D-glucose, 0.5; D-mannitol, 10.0; aesculin, 0.8; iron(III)ammonium citrate, 0.5; phenol red, 0.08; polymyxin B (IU), 100 000; acriflavine HCl, 0.005; lithium chloride, 15.0; ceftazidime (may be replaced by Moxalactam), 0.02; distilled or deionized water, 1000.0. Incubation was performed at 30 °C for 24 and 48 h microaerobically in a jar containing 5–12% CO₂, 5–15% O₂, and 75% N₂. Two kinds of PALCAM agar were tested. No. 1 was prepared from the ingredients by ourselves, No. 2 was the original Merck medium.

Acriflavine-ceftazidime (AC) agar [10]. Columbia agar base (Gibco), 44.0; acriflavine HCl, 0.01; ceftazidime pentahydrate (Glaxo), 0.05; distilled or deionized water, 1000.0. Incubation at 35 °C for 48 h.

Oxford agar [11]. Columbia agar base, 39.0; aesculin, 1.0; iron(III)ammonium citrate, 0.5; lithium chloride, 15.0; cycloheximide, 0.4; colistin, 0.02; acriflavine (Sigma), 0.005; cefotetan, 0.002; fosfomycin, 0.01; distilled or deionized water, 1000.0. Incubation lasted at 30 °C for 48 h.

Tripaflavine-nalidixic acid-serum agar (TNSA) [12, 13]. Lab-Lemco powder (Oxoid), 10.0; Bacto peptone (Oxoid), 10.0; yeast extract, 3.0; soluble haemoglobin powder (Oxoid), 0.1; sodium chloride, 5.0; acriflavine (Trypaflavine, Reanal, Budapest, Hungary), 0.012; nalidixic acid, 0.04; bovine serum (ml), 50.0; Bacto agar (Difco), 20.0; distilled or deionized water, 900.0. Incubation at 37 °C for 24 h and at room temperature for another day.

Furray's medium [14]. Corbovin (Meat extract, Human, Budapest, Hungary), 5.0; tryptone, 5.0; yeast extract, 5.0; sodium chloride, 5.0; agar, 15.0; oxolinic acid, 0.03; xantacrine (Trypaflavine, Reanal, Budapest, Hungary), 0.005. The ingredients except oxolinic acid and tripaflavine were suspended in water and sterilized at 121 °C for 15 min. After cooling to 50 °C 10 ml of oxolinic acid (0.150 g in 50 ml distilled water sterilized by filtration) and 1 ml of tripaflavine (0.250 g in 50 ml distilled water sterilized by filtration) were added. Incubation lasted at 37 °C for one day and at room temperature until the second day.

University of Vermont (UVM) broths [5]. Proteose peptone (Difco), 5.0; tryptone (Difco), 5.0; yeast extract, 5.0; Lab-Lemco powder (Oxoid), 5.0; sodium chloride, 20.0; Na₂HPO₄ · 2H₂O, 12.0; KH₂PO₄, 2.35; aesculin (Sigma), 1.0; nalidixic acid (Sigma), 0.04; acriflavine HCl (Sigma), 0.012 or 0.025; distilled or deionized water, 960.0. Aqueous solution of 0.012 g acriflavine (UVM I) or 0.025 g acriflavine (UVM II) was added to the cooled base.

Specimens. Forty-three sausages and 103 raw milks were sampled and examined for the presence of *Listeria*. Raw milk samples (25 ml) were cultivated in 225 ml Levinthal broth with acriflavine, nalidixic acid and blood at 4 °C for 3 months [7]. pH of the cultures was frequently checked and when under 6.4 it was readjusted to 7.0 with alkali.

Primary enrichment is accomplished by mixing 25 g of sausage with 225 ml of UVM I. The mixture is incubated at 30 °C. After 24 h incubation UVM I was spread on LPM agar and 0.1 ml of UVM I is added to 10 ml UVM II and incubated for 24 h.

Strains. Sixteen strains were used for the model experiments. These are the following. *Listeria monocytogenes* serovar 1/2a ATCC 19111/NCTC 7973, serovar 1/2c ATCC 19112/NCTC 5348, serovar 3a ATCC 19113/NCTC 5105, serovar 4a ATCC 19114/NCTC 5214, serovar 4b NCTC 4885, serovar 6 NCTC 10889/SLCC 2480, *Listeria ivanovii* serovar 5 ATCC 19119/NCTC 11846/SLCC 2379, *Listeria grayi* subsp. *grayi* L.332/64, *Bacillus cereus* ATCC 9634, *Brochotrix thermosphacta* HNCMB 139001, *Lactobacillus plantarum* ATCC 8014, *Micrococcus luteus* ATCC 9341, *Pseudomonas aeruginosa* HNCMB 170001, *Proteus vulgaris* HNCMB 60001, *Staphylococcus aureus* ATCC 9144, *Enterococcus faecalis* NCTC 8213.

Procedure. The 7 media were controlled in accordance to the "Testing methods for use in quality assurance of culture media" [15]. Modified Miles-Misra method was used. The strains were cultivated in broth for 18 h at 37 °C. Aliquots from ten-fold dilutions of the strains were dropped onto the media. The plates were incubated as described above. The number of colonies was counted using the oblique light technique. Productivity and selectivity of the media were calculated on the basis of the colony counts determined on the different

media. The colony count on blood agar was the basis for the calculation of the ratios. The experiments were repeated minimum three times. $PR_s = N_s/N_0$ where N_s = colony count on the medium tested; N_0 = colony count on the reference medium.

Results and discussion

In the tables results of 3, 4 or 5 comparable studies are summarized. First the productivity of the media was determined. On the basis of the results it can be concluded that Oxford medium was the best among the 7 media studied. LPM agar, AC agar and Forray's medium were more inhibitory for one or two *Listeria* strains. Neither TNSA medium nor PALCAM agars (1 and 2) were as suitable as the aforementioned four media. The growth of some *Listeria* strains were more or less inhibited on them. In case of PALCAM agars some *Listeria* strains grew very slowly and their colonies appeared on the media only after 2-3 days.

Table I
The productivity ratios of different Listeria strains

Strains		Blood agar	Oxford agar	LPM agar	AC agar	TNSA agar	PALCAM agar No. 1
<i>L. monocytogenes</i>	1/2a	1.000	0.891	0.120	0.900	0.440	0.000
	1/2c	1.000	0.713	0.638	0.725	0.472	0.401
	3a	1.000	0.773	0.418	0.0014	0.134	0.0014*
	4a	1.000	0.398	0.551	0.478	0.0074	0.0052*
	4b	1.000	0.708	0.556	0.332	0.0088	0.190
	6	1.000	0.936	1.031	0.926	0.377	0.819
<i>L. ivanovii</i>	5	1.000	0.319	0.318	0.288	0.0073	0.030*
<i>L. grayi</i>		1.000	0.000	0.000	0.000	0.000	0.000

* After 48 h

Table II
The productivity ratios of different Listeria strains

Strains		Blood agar	LPM agar	Forray's medium
<i>L. monocytogenes</i>	1/2a	1.000	0.030	0.234
	1/2c	1.000	0.608	0.086
	3a	1.000	0.494	0.215
	4a	1.000	0.562	0.112
	4b	1.000	0.711	0.754
	6	1.000	0.577	0.372
<i>L. ivanovii</i>	5	1.000	0.298	0.618
<i>L. grayi</i>		1.000	0.000	0.000

It is interesting to mention that during the different experiments alterations could also be observed in the growth ratios, size and morphology of colonies and intensity of growth of a given *Listeria* strain on the same medium (see the Tables). AC agar was less selective to *Brochotrix thermosphacta* than the other media (Table IV).

Table III

The productivity ratios of different Listeria strains

Strains		Blood agar	LPM agar	PALCAM agar No. 2
<i>L. monocytogenes</i>	1/2a	1.000	0.0005	0.0004*
	1/2c	1.000	0.873	0.632
	3a	1.000	0.909	0.003
	4a	1.000	0.380	0.229
	4b	1.000	0.066	0.292
	6	1.000	0.893	0.851
<i>L. ivanovii</i>	5	1.000	1.020	0.090
<i>L. grayi</i>		1.000	0.000	0.000

* After 48 h

Parallel with these comparative studies samples of 43 different sausages which were enriched in UVM broths were subcultured onto Oxford, LPM, AC, TNSA media and PALCAM agar No. 1. The number of positive samples was 43 on Oxford, LPM, AC and TNSA media but less on PALCAM agar No. 1.

Table IV

Selective effect of media expressed by productivity ratios

Strains	Blood agar	Oxford agar	LPM agar	AC agar	TNSA agar	PALCAM agar No. 1
<i>P. aeruginosa</i>	1.000	0.000	0.000	0.000	0.000	0.000
<i>S. faecalis</i>	1.000	0.000	0.000	0.000	0.000	0.000
<i>M. luteus</i>	1.000	0.000	0.000	0.000	0.000	0.000
<i>L. plantarum</i>	1.000	0.000	0.000	0.000	0.000	0.000
<i>B. thermosphacta</i>	1.000	0.000	0.000	0.159	0.000	0.000
<i>P. vulgaris</i>	1.000	0.000	0.000	0.000	0.000	0.000
<i>S. aureus</i>	1.000	0.000	0.000	0.000	0.000	0.000
<i>B. cereus</i>	1.000	0.000	0.000	0.000	0.000	0.000

When 103 milk samples, after the cold enrichment, were subcultured on TNSA and PALCAM agar No. 2, the number of listeria positive samples was the same on both media but the latter one was more inhibitory and more selective [16].

In conclusion, the use of Oxford agar can currently be recommended. Oxford agar ensures excellent conditions for the early and intensive growth of

Listeria strains. LPM agar was inhibitory against serotype 1/2a and some *Listeria* strains formed too small colonies after one day incubation on this medium. The productivity values of AC agar were similar to that of LPM but it inhibited the growth of serovar 3 but not serovar 1/2a. These media were followed by Forray's agar, TNSA and PALCAM agar No. 1 and 2.

Using Henry's method on LPM agar, in contrast with Oxford agar, different types of colony morphology of *Listeria* strains could be recognized. The same phenomenon could be observed on TNSA medium, too. So, application of Henry's method for isolation of *Listeria* strains gives a greater possibility to recognize different variety of species, serotypes and virulence of that bacteria.

The causes of alterations in productivity observed on the same medium in the repeated experiments are not known.

Acknowledgements. The authors are greatly indebted to G. D. W. CURTIS (Oxford, England) as well as W. GIELEN (the representative of Oxoid Limited Basingstoke, England) for Oxford medium, T. GESZTES (the representative of Glaxo Operations UK Ltd., Greenford, England) for Ceftazidime, W. SCHMIDT (the representative of Merck, Darmstadt, Germany) for PALCAM medium, M. RÁSKAI (Human, Budapest, Hungary) for Corbovin and Oxolinic acid and to B. LÁNYI (National Institute of Hygiene, Budapest, Hungary) for the HNCMB strains.

REFERENCES

1. Anonymous: *Acta Microbiol Hung* **36**, 79 (1989).
2. Anonymous: Foodborne listeriosis. Report of a WHO Informal Working Group. Geneva 1988.
3. Baird, R. M., Corry, J. E. L., Curtis, G. D. W., Mossel, D. A. A., Skovgaard, N. P.: *Internat J Food Microbiol* **9**, iii (1989).
4. Fraser, J. A., Sperber, W. H.: *J Food Prot* **51**, 762 (1988).
5. McClain, D., Lee, W. H.: Laboratory Communication No. 57. Revised May 24, 1989.
6. Anonymous: *Internat J Food Microbiol* **8**, iii (1989).
7. Ralovich, B.: *Listeriosis Research; Present Situation and Perspective*. Akadémiai Kiadó, Budapest 1984.
8. Lee, W. H., McClain, D.: *Appl Environ Microbiol* **52**, 1215 (1986).
9. Van Netten, P., Perales, I., van de Moosdijk, A., Curtis, G. D. W., Mossel, D. A. A.: *Internat J Food Microbiol* **8**, 299 (1989).
10. Bannerman, E. S., Bille, J.: *Appl Environ Microbiol* **54**, 165 (1988).
11. Curtis, G. D. W., Mitchell, R. G., King, A. F., Griffin, E. J.: *Lett Appl Microbiol* **8**, 95 (1989).
12. Ralovich, B., Forray, A., Mérő, E., Málovics, I., Százados, I.: *Egészségtudomány* **14**, 305 (1970).
13. Ralovich, B., Forray, A., Mérő, E., Málovics, I., Százados, I.: *Zentralbl Bakteriell [A]* **216**, 88 (1971).
14. Forray, A.: Personal communication.
15. Anonymous: *Internat J Food Microbiol* **5**, 291 (1987).
16. Ibrahim, G. A. M.: Personal communication.

WHAT IS THE DIAGNOSTIC VALUE OF BETA-D-GLUCURONIDASE (BDG) ACTIVITY OF BACTERIA USING FLUOROCULT ECD AGAR FOR THEIR CULTIVATION?

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(Received January 17, 1991)

A total of 1510 strains from 15 genera of Gram-negative and Gram-positive bacteria were studied. More than 94% of 327 *Escherichia coli* strains showed beta-D-glucuronidase (BDG) activity. Seventeen serotypes from 170 *E. coli* O serogroup representatives were negative. Relationship between the existence of BDG positive and negative *E. coli* strains in the same serogroup or serotype has not been observed. The rate of BDG positivity was 42% among *Salmonella arizonae* strains and 42.2% among *Shigella* strains. Only one *Citrobacter* strain out of the 971 strains belonging to *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Proteus*, *Serratia*, *Yersinia*, *Pseudomonas*, *Aeromonas*, *Vibrio* and *Listeria* was BDG positive. A screening method based on only BDG activity is not sufficient for the primary diagnosis of *E. coli*.

As coliforms, faecal coliforms, faecal coli and *Escherichia coli* strains have been considered indicator of faecal contamination, their detection and enumeration in food, water and environmental samples are very important. They can be detected on plates and/or in tubes (Most Probable Number) [1–4]. Both methods are laborious and time-consuming. In addition, these methods have been reported to be somewhat inadequate as single *E. coli* strains may be sensitive to the higher incubation temperature and to the selective media used [4–8]. Besides, false positive and negative results may occur in consequence of bacterial interference, and the late lactose fermentors may not be detected during the two days long incubation [4, 6, 7].

There has been a tendency to use enzyme profiles of bacteria for their detection and identification long ago. Widely different compounds (enzyme substrates) were conjugated with chromogenic or fluorogenic material to get a colourless substance for the degradation by bacterial enzyme releasing the colour or fluorescent material [9–16].

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Existence of BDG activity of microbes was supposed during the 1930's and verified later on. Taxonomic and diagnostic importance of BDG activity has been studied by numerous researchers. As the majority of *E. coli* strains possess BDG activity, the use of this enzyme activity has been proposed for rapid identification or easy detection of these bacteria. In contrast, some authors published that *E. coli* O157:H7 strains are BDG negative and Chang et al. [17] emphasized the need for caution in using MUG-plate for the detection of faecal *E. coli*. We observed other BDG negative *E. coli* serotypes [18]. The aim of our present study was to examine theoretical and practical aspects of BDG activity of different bacteria in the primary diagnostic work.

Materials and methods

Strains. One *Aeromonas*, 65 *Citrobacter* (*C. freundii* O1-O42 type strains, 1 *C. diversus* and others), 1 *Edwardsiella*, 117 *Klebsiella*, (61 *K. pneumoniae*, 12 *K. oxytoca*, 5 *K. ozaenae*, 5 *K. aerogenes* and 34 others), 58 *Enterobacter cloacae* O group strains, 327 *E. coli* (O1-O170, K1-K88, H1-H56 reference strains and others), 51 *Hafnia alvei* O group strains, 591 *Proteaceae* (138 *P. vulgaris*, 343 *P. mirabilis*, 49 *Morganella morganii*, 44 *Providencia rettgeri*, 17 *P. inconspicua* O group strains and others), 40 *Pseudomonas aeruginosa* O group strains, 69 *Salmonella arizonae* O group strains and others, 4 *Serratia* (3 *S. marcescens*, 1 *S. liquefaciens*), 142 *Shigella* (*S. dysenteriae*, *S. boydii*, *S. flexneri* and *S. sonnei* reference strains and others), 1 *Vibrio* (non-O1) and 2 *Yersinia enterocolitica* strains from the Hungarian National Collection of Medical Bacteria, National Institute of Hygiene, Budapest, Hungary. Further 24 *Vibrio* strains (10 *V. parahaemolyticus*, 9 *V. alginolyticus* and *V. cholerae* non-O1, *V. fluvialis*, *V. furnissii*, *V. harveyi* and *V. vulnificus*, one strain of each) from T. Miyamoto, Kyushu University, Fukuoka 812, Japan, and 16 freshly isolated *Listeria* strains from our own collection.

BDG activity of the strains was assessed on Fluorocult ECD Agar (Merck Cat. No. 403B). Agar culture of the strains was used for the test. The ECD Agar plates were incubated at 37 °C for two days. The fluorescence was examined by UV lamp (Merck Cat. No. 13203) at 360 nm daily.

Biochemical reactions were performed by conventional methods.

Virulence of enteroinvasive *E. coli* strains was tested by the Serény test [19].

Results

First BDG activity of 1494 cultures belonging to 14 genera of Gram-negative microorganisms and that of 16 *Listeria* strains were studied. To get more reliable results when it was possible O, K and H antigen reference strains were tested. The results are presented in Tables I, II and III. Summarizing our results in Table I, *E. coli* strains were positive in 94.8%, *S. arizonae* strains in 42.0% and *Shigella* strains in 42.2%. Out of 971 strains belonging to 12 other genera, only one *Citrobacter* strain was positive. BDG positivity of different *Shigella* species was not the same. All *S. sonnei* strains were positive whereas 63.8% of *S. boydii*, 42.1% of *S. dysenteriae* and 20.3% of *S. flexneri* strains showed BDG activity. Seventeen serotypes (O8:K46:H30 ETEC?, O19b:K.:H7, O20a, 20b:K84:H26 ETEC?, O25:K11:H6 ETEC?, O55:K59:H.

Table I

Beta-D-glucuronidase activity of Gram-negative and Gram-positive bacteria

Genera	Strains and authors						
	Present work		Other authors (summarized) [6, 10, 15, 31, 32, 35, 41]		Total		
	No. of strains	No. of positive strains	No. of strains	No. of positive strains	No. of strains	No. of positive strains	%
<i>Citrobacter</i>	65	1	128	3	193	4	2.0
<i>Edwardsiella</i>	1	—	6	—	7	—	.
<i>Enterobacter</i>	58	—	233	1	291	1	0.3
<i>Escherichia</i>	327	310	2068	1942	2395	2252	94.0
<i>Hafnia</i>	51	—	60	—	111	—	0.0
<i>Klebsiella</i>	117	—	523	3	640	3	0.4
<i>Proteus</i>	591	—	263	—	854	—	0.0
<i>Salmonella</i>	.	.	749	275	749	277	36.9
<i>Arizona</i>	69	29	.	.	69	29	42.0
<i>Serratia</i>	4	—	92	3	96	3	3.1
<i>Shigella</i>	142	60	382	207	524	267	50.9
<i>Yersinia</i>	2	—	60	6	62	6	9.6
<i>Pseudomonas</i>	40	—	74	2	114	2	1.7
<i>Aeromonas</i>	1	—	134	—	135	—	0.0
<i>Vibrio</i>	25	—	45	—	70	—	0.0
<i>Erwinia</i>	.	.	14	—	14	—	0.0
<i>Alcaligenes</i>	.	.	2	—	2	—	.
<i>Acinetobacter</i>	.	.	2	—	2	—	.
<i>Moraxella</i>	.	.	1	—	1	—	.
<i>Staphylococcus</i>	.	.	238	21	238	21	8.8
<i>Streptococcus</i>	.	.	1168	551	1168	551	47.1
<i>Clostridium</i>	.	.	197	121	197	121	61.4
<i>Listeria</i>	16	—	.	.	16	—	.

EPEC?, O60:K.:H-, O63:K.:H-, O78:K80:H- ETEC?, O82:K.:H-, O89:K.:H16, O91:K.:H-, O93:K.:H8, O108:K.:H10, O120:K.:H6, O124:K72:H30 EIEC, O136:K78:H- and O163:K.:H19) out of the 170 *E. coli* serogroup representatives proved to be BDG negative. In the following different serotypes of the same *E. coli* serogroups were examined to recognize whether BDG positive and negative serotypes belonged to the same serogroup. Twenty-three serotypes of three serogroups were controlled and each of them contained one or more BDG negative strains besides the positive ones (O1:K51:H-BDG⁺, O1:K.:HNT BDG⁻, O1:K1:HNT BDG⁺, O8:K.:H9 BDG⁺, O8:K8:H4 BDG⁺, O8:K.:H21 BDG⁺, O8:K.:H20 BDG⁺, O8:K25:H9 BDG⁺, O8:K27:H- BDG⁺, O8:K40:H9 BDG⁺, O8:K41:H11 BDG⁺, O8:K42:H-BDG⁺, O8:K43:H11 BDG⁺, O8:K44:H- BDG⁺, O8:K45:H9 BDG⁺, O8:K46:H30 BDG⁻, O8:K47:H2 BDG⁺, O8:K48:H9 BDG⁺, O8:K49:H21 BDG⁺, O8:K50:H- BDG⁺, O124:K72:H- BDG⁺ (3 strains), O124:K72:HNT BDG⁻ (5 strains) and O124:K72:H30 BDG⁻).

Table II

Beta-D-glucuronidase activity of E. coli strains of late lactose fermenters

Designations of strains	Serotype of strains	Time of lactose fermentation in days	Beta-D- glucuronidase test
228/89	O1:K.:HNT	3	—
740/1/89	O4:K.:H1	5	+
743/89/1	O4:K.:H1	5	+
1136/89	O4:K.:H5	4	+
1127/89	O4:K.:H1	4	+
1129/89	O4:K.:H1	6	+
917/88/1	O6:K.:H1	3	+
917/88/2	O6:K.:H1	3	+
383/89	O6:K.:H1	6	+
640/89	O6:K.:H1	4	+
1139/89	O6:K.:H1	6	+
345/89	O8:K.:H9	>14	+
974/89/1	O124:K72:H-	3	+
865/89	O124:K72:HNT	3	—
766/89	O124:K72:H-	4	+
569/89*	O124:K72:HNT	3	—
568/89*	O124:K72:HNT	3	—
567/89*	O124:K72:HNT	3	—
566/89*	O124:K72:HNT	3	—
258/89	O144:K.:H-	5	+
890/89	O144:K.:H-	4	+
1115/89	O151:K.:HNT	11	+
1116/89	O151:K.:HNT	11	+
1110/89	O151:K.:HNT	>14	+
374/89	ONT:K.:H27	3	—
672/89	Osp:K.:H-	14	—

* These strains were isolated from the same outbreak
K. = K antigen not determined

Finally, the relationship between late lactose fermentation and BDG activity of *E. coli* strains was studied. The results are presented in Table II. Out of 26 late lactose fermenters only 7 strains were BDG negative. So it seems that the result of BDG test was not influenced by the character of lactose splitting. On the other hand, some relationship might exist between virulence and BDG activity of entero invasive *E. coli* strains.

Discussion

Enzyme activity of bacteria has long been used for their identification and classification. During the 1930's it was supposed that microorganisms might possess BDG activity [20]. Later Patterson [21], Bucher and Geschickter [22] as well as Barber et al. [23] verified that sexual hormones conjugated with glucuronic acid could be hydrolyzed by bacteria. Buehler et al. [24, 25] pre-

Table III

Beta-D-glucuronidase activity of Shigella strains

Species/Serotypes	Present work			Massenti et al. [31]			Kilian and Bülow [28]			Total no. of strains	Positivity %
	No. of strains	No. of positive strains	%	No. of strains	No. of positive strains	%	No. of strains	No. of positive strains	%		
<i>S. sonnei</i>	3	3	.	52	52	100	63	62	98	118	99.1
<i>S. dysenteriae</i>	1	6	0	7	0					13	
	2	11	0	15	0					26	
	3	3	3	6	6					9	
	4	3	2	4	4					7	
	5	3	0	4	0					7	
	6	3	3	3	3					6	
	7	2	2	2	2					4	
	8	2	2	5	5					7	
	9	1	0							1	
	10	1	1							1	
	n.t.	3	3							3	
Total	38	16	42.1	46	20	43.4	13	7	54	97	44.3
<i>S. boydii</i>	1	3	3	10	10					13	
	2	3	0	8	0					11	
	3	3	2	6	6					9	
	4	2	0	8	0					10	
	5	6	5	7	7					13	
	6	3	2	5	5					8	
	7	3	2	10	10					13	
	8	3	2	3	3					6	
	9	2	2	2	2					4	
	10	3	3	4	4					7	
	11	3	0	4	2					7	
	12	2	2	1	1					3	
	13	3	0	4	0					7	
	14	3	2	1	1					4	
	15	3	3	1	1					4	
	n.t.	2	2							2	
Total	47	30	63.8	74	52	70.2	6	1	17	127	65.3
<i>S. flexneri</i>	1a	3	0	28	0					31	
	1b	3	0	15	0					18	
	2a	6	0	22	0					28	
	2b	3	0	3	0					6	
	3a	4	3	18	18					26	
	3b	1	0								
	3 degr.	3	1								
	4a	9	4	6	6					15	
	4b	4	0	4	0					8	
	5	6	0	9	0					15	
	6	4	1	22	17					26	
	x	3	1							3	
	y	5	1							5	
Total	54	11	20.3	127	41	32.2	41	12	29	222	28.8
Total	142	60	42.2	299	165	55.1	123	82	67	564	54.4

pared and studied beta-glucuronidase from *E. coli*. Mead et al. [26] described a method using 4-methylumbelliferone for fluorometric demonstration of this enzyme. Dahlén and Linde [27] developed MUG-plate for screening bacterial BDG activity. Maddocks and Greenan [9] used filter paper strips method. Kilian and Bülow [28] proposed to use PGUA agar to detect glucuronidase activity of enteric bacteria. Le Minor et al. [11] and Le Minor [12] applied a disk method. Feng and Hartman [6] and Edberg and Kontnick [29] used fluorometric and colorimetric tube, plate and disk methods. Papasian and Hertlein [30] evaluated three fluorogenic methods. The methods included: direct disk inoculation test, tube test and liquid spot reagent test. Delisle and Ley [15] developed a new chromogenic compound for rapid detection of *E. coli* from urine on plate. Thompson et al. [16] applied RM-MUG reagent. Taxonomic and diagnostic importance of beta-glucuronidase activity of microorganisms has been studied since the 1950's [5–8, 10–12, 15, 17, 18, 28, 30–42].

Our observations and the results of different authors are summarized in Tables I, II and III. For *E. coli* other authors found positive results between 93.2% and 96.6%, only Edberg and Kontnick [29] and Delisle and Ley [15] observed less than 90% positivity (88%, and 83.8–89.4% with two different methods, respectively). In the case of *Salmonella* strains Le Minor et al. [11] examined 4114 cultures 1241 (10.1%) of which exerted BDG activity. Massenti et al. [31] studied 565 different *Salmonella* strains and obtained 42.6% positivity. The results of the others were between 0% and 25%. Our result for salmonellae of group *S. arizonae* is similar to that of Massenti et al. [31]. As to *Shigella* strains the rate of positivity was similar to ours in case of Hartman [35] — 44.1%, but Kilian and Bülow [28] and Massenti et al. [31] observed higher rates — 56.8% and 55.1%, respectively. It is clear that the rate of positivity is not the same in case of single *Shigella* species. Comparing our results with those of Killian and Bülow [28] and Massenti et al. [31] the percentages are the following: *S. sonnei* 100, 98 and 100%; *S. dysenteriae* 42.1, 54 and 43.4%; *S. boydii* 63.8%, 17% and 70.2% and finally *S. flexneri* 20.3%, 29% and 32.2%. Also, the positivity was not the same in case of different serotypes of the single *Shigella* species. Our observations and the results of Massenti et al. [31] are quite similar. At present there is no explanation for the cause and the importance of this phenomenon. As to the other genera, only 1 *Citrobacter* strain out of the 971 strains was BDG positive in our study. In the case of *Citrobacter* strains 4 positive (3 by Hartman [35] and 1 by us), of *Enterobacter* isolates 1 positive, of *Klebsiella* cultures 3 positive and of *Serratia* strains 3 positive isolates were observed up to now [35]. Among *Yersinia* isolates 6 strains [31] and finally among *Pseudomonas* strains 2 cultures were positive [41]. Petzel and Hartman [7] also observed BDG positivity among *Pseudomonas* and *Flavobacterium* strains cultured from vegetables. At present there are some genera — *Edwardsiella*, *Hafnia*, *Serratia*, *Yersinia*, *Pseudo-*

monas, *Aeromonas*, *Vibrio*, *Erwinia* and *Listeria* — in case of which it is not possible to give the exact rate of positivity because the number of the strains is small. We observed that at least 17 strains out of the 170 *E. coli* serogroup representatives were negative. One serotype out of the 17 is undoubtedly associated with human diarrhoeal diseases: *E. coli* O124:K72:H30 which is a well-known enteroinvasive organism [43]. Five other serotypes may perhaps be in connection with diarrhoeal events, too. These are: O8:K46:H30, O20ab:K84:H26, O25:K11:H6, O55:K59:H- and O78:K80:H-. The other 11 BDG negative strains are not associated with enteric diseases. First Doyle and Schoeni [5] gave the exact serotype of a BDG negative pathogenic *E. coli* strain. That was the enterohaemorrhagic *E. coli* O157:H7. Later this observation was verified by others [8, 36, 39, 41]. Krishnan et al. [36] published that three *E. coli* O111:K58 strains and one O1 strain were BDG negative. We also found a BDG negative *E. coli* O1 strain. Bülte and Reuter [41] had one BDG negative ETEC strain. Chang et al. [17] frequently isolated BDG negative *E. coli* strains from the faeces of healthy persons and rats; accordingly, they think that BDG may be a convenient tool for detecting some *E. coli* but it cannot be used for the demonstration of faecal coli. As the frequency of BDG positive and negative *E. coli* serotypes in the same serogroup has not yet been investigated, we tested 23 serotypes of three serogroups. Each of the serogroups contained one or more BDG negative serotypes besides the positive ones, i.e. BDG positive and negative *E. coli* strains may belong to the same serotype.

The results indicate that a screening method based on only BDG activity of *E. coli* strains cannot be used even for a primary diagnosis of human enteric infections or for the demonstration of some *E. coli* strains associated with human diarrhoeal diseases, or of faecal coli in food and water. That only 7 strains out of the 26 late lactose fermenter *E. coli* strains (Table II) were BDG negative, indicates that the result of BDG test was not influenced by the character of lactose splitting. Therefore, the late lactose fermenters which may not be detected by the classical plate and tube methods [1-4] at least partly can be detected and enumerated with the help of BDG activity.

When the virulence — keratoconjunctivitis causing capacity — of enteroinvasive *E. coli* strains was compared with the result of BDG test, it could be supposed that some relationship might exist between these characters. Our assumption is under examination. Recently Thompson et al. [16] published a connection existing between verocytotoxin production and RM-MUG negativity of *E. coli* O157 strains.

Acknowledgements. The authors wish to thank Mr. W. E. SCHMIDT (Productmanager, E. Merck, Darmstadt, Germany) for Fluorocult ECD Agar and UV lamp, and they are also grateful to Mr. T. MIYAMOTO (Kyushu University, Fukuoka 812, Japan) for the 24 *Vibrio* strains.

REFERENCES

1. The International Standards Organization (ISO) 7251 (1984).
2. The International Standard Organization (ISO) 4831 (1978).
3. The International Standards Organization (ISO/DIS) 6391 (1985).
4. Hofstra, H., Huisin't Veld, J. H. J.: *J Appl Bacteriol Symp Suppl* **65**, 197S (1988).
5. Doyle, M. P., Schoeni, J. L.: *Appl Environ Microbiol* **48**, 855 (1984).
6. Feng, P. C. S., Hartman, P. A.: *Appl Environ Microbiol* **43**, 1320 (1982).
7. Petzel, J. P., Hartman, P. A.: *Appl Environ Microbiol* **49**, 925 (1985).
8. Szabó, R. A., Todd, E. C. D., Jean, A.: *J Food Prot* **49**, 768 (1986).
9. Maddocks, J. L., Greenan, M. J.: *J Clin Path* **28**, 686 (1975).
10. Kilian, M., Bülow, P.: *Acta Path Microbiol Scand Sect B* **84**, 245 (1976).
11. Le Minor, L., Buisnière, J., Novel, G., Novel, M.: *Ann Microbiol (Paris)* **B 129**, 155 (1978).
12. Le Minor, L.: *Zentralbl Bakteriell [A]* **243**, 321 (1979).
13. Miyamoto, T., Sheu, Y. I., Miwa, H., Hatano, S.: *J Food Hyg Soc Japan* **30**, 534 (1989).
14. Miyamoto, T., Miwa, H., Hatano, S.: *Appl Environ Microbiol* **56**, 1480 (1990).
15. Delisle, G. J., Ley, A.: *J Clin Microbiol* **27**, 778 (1989).
16. Thompson, J. S., Hodge, D. S., Borczyk, A. A.: *J Clin Microbiol* **28**, 2165 (1990).
17. Chang, G. W., Brill, J., Lum, R.: *Appl Environ Microbiol* **55**, 335 (1989).
18. Ibrahim, G. A. M., Fábíán, A., Herpay, M., Ralovich, B.: In *IUMS C, Osaka, '90, Program and Abstracts, Osaka 1990*. PS75-17. p. 112.
19. Serény, B.: *Acta Microbiol Acad Sci Hung* **2**, 293 (1955).
20. Cohen, S. L., Marrian, G. F.: *Biochem J* **28**, 1603 (1934).
21. Patterson, J.: *Br Med J* **2**, 522 (1937).
22. Bucher, N. L. R., Geschickter, C. F.: *Endocrinology* **27**, 727 (1940).
23. Barber, M., Brooksbank, B. W. L., Haslewood, G. A. D.: *Nature* **162**, 701 (1948).
24. Buehler, H. J., Katzman, P. A., Doisy, E. A.: *Fed Proc* **8**, 189 (1949).
25. Buehler, H. J., Katzman, P. A., Doisy, E. A.: *Proc Soc Exp Biol Med* **76**, 672 (1951).
26. Mead, J. A. R., Smith, J. N., Williams, R. T.: *Biochem J* **61**, 569 (1955).
27. Dahlén, G., Linde, A.: *Appl Microbiol* **26**, 863 (1973).
28. Kilian, M., Bülow, P.: *Acta Path Microbiol Scand Sect B* **87**, 271 (1979).
29. Edberg, S. C., Kontnick, C. M.: *J Clin Microbiol* **24**, 368 (1986).
30. Papasian, C. J., Hertlein, G.: *Diagn Microbiol Infect Dis* **8**, 255 (1987).
31. Massenti, M. F., Scarlata, G., Nastasi, A.: *Boll Ist Sieroter Milan* **60**, 26 (1981).
32. Hansen, W., Yourassowsky, P. A.: *J Clin Microbiol* **20**, 1177 (1984).
33. Robison, B. J.: *Appl Environ Microbiol* **48**, 285 (1984).
34. Moberg, L. J.: *Appl Environ Microbiol* **50**, 1383 (1985).
35. Hartman, P. A.: In *Fifth Internat Symposium on Rapid Methods and Automation in Microbiology, Florenz* (1987).
36. Krishnan, C., Fitzgerald, V. A., Dakin, S. J., Behme, R. J.: *J Clin Microbiol* **25**, 1043 (1987).
37. Heizmann, W., Döller, P. C., Gutbrod, B., Werner, H.: *J Clin Microbiol* **26**, 2682 (1988).
38. Moberg, L. J., Wagner, M. K., Kellen, L. A.: *J Assoc Off Anal Chem* **71**, 589 (1988).
39. Ratnam, S., March, S. B., Ahmed, R., Bezanson, G. S., Kasatiya, S.: *J Clin Microbiol* **26**, 2006 (1988).
40. Bülte, M., Reuter, G.: *Arch Lebensmittelhyg* **40**, 126 (1989).
41. Bülte, M., Reuter, G.: *Zentralbl Hyg* **186**, 284 (1989).
42. Hofmann, G., Schwien, U.: *Fluoreszenzoptischer Nachweis von E. coli, Grundlagen und Praxis, Merck-Seminar, Würzburg 1988*. GIT Verlag, Darmstadt 1989.
43. Farmer, J. J. III., Wels, M. S., Griffin, P. M., Wachsmuth, I. K.: In *Diagnostic Procedures for Bacterial Infections*, Wentworth Bertina, B. (ed.) 7th Ed. A.P.H.A., Inc Washington 1987. p. 233.

MEDITATION OF THE TAXONOMY OF *LISTERIA**

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(Received February 4, 1991)

Listeria monocytogenes was exactly identified and named in 1924. Since then the name and taxonomic position of listeriae have been changed several times. The last classification was performed on the basis of genetic studies and of some biological properties (haemolytic character, virulence and acid production from sugars). On the basis of the taxonomic validity of these characters, the *Listeria* genus is proposed to be classified into three species: *L. monocytogenes* (four subspecies), *L. ivanovii* and *L. grayi* (two subspecies).

Until recently bacteria were classified into species and genera only on the basis of their phenotypic characters, namely morphological, tincrorial, biochemical and serological properties. Listeriae were identified and classified mainly in this way between 1924 and 1975 [1–17]. The results of different efforts can be seen in Table I. During this period of time, and even afterwards, subjective impressions influenced the results of classification. The decision of Wilson and Miles [18], Breed et al. [6] and Prévot [2] is a good example of this.

In the 1960's new techniques were evolved in consequence of which the classical bacterial taxonomy has been changed. The basis of the new classification is DNA relatedness and GC percentage of the bacterial DNA. Recently the results of 16S r RNA cataloguing studies are also used. Besides these genetic methods, Gram staining and biochemical tests have kept their taxonomic importance, too, and new probes have also been applied. So, lysis by specific phages or determination of susceptibility or resistance of bacteria to different kinds of substances have been used. However, the use of commercial identification systems has modified the evaluation of the results of biochemical tests; the incubation period has become shorter: two days only. When studying *Enterobacteriaceae*, CDC researchers used also two-day results. Two types of computer programmes have been developed. One is the normalized likelihood programme, the other is the strain matcher programme [19].

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* This paper was partly presented on the Microbiology Seminar of J. Radcliffe Hospital, in Oxford, UK, 19th June, 1990, and on the Meeting of ICSB, Subcommittee on *Listeria* in Osaka, Japan, 17th September, 1990.

Table I

Progress in the classification of *Listeria* strains during 1924-1971

Year	Species/subspecies	Genus	Family	References
1924	<i>Bacterium monocytogenes</i>			Murray, Webb, Swann [1]
1927	<i>Listerella hepatolytica</i>	<i>Listerella</i>		Pirie [38]
1930s	<i>Listerella monocytogenes</i>	<i>Listerella</i>		
1940	<i>Listeria monocytogenes</i>	<i>Listeria</i>		Pirie [16]
1948	L ²⁶ strain	<i>Listeria</i>		Sohier, Benazet, Piechaud [15]
1954	<i>Erysipelothrix monocytogenes</i>	<i>Erysipelothrix</i>		Wilson, Miles [18]
1957	<i>L. monocytogenes</i>	<i>Listeria</i>	<i>Corynebacteriaceae</i>	Breed, Murray, Smith [6]
1957	<i>L. monocytogenes</i>	<i>Listeria</i>	<i>Bacteriaceae</i>	Prévot [2]
1961	<i>L. denitrificans</i> (L ²⁶)	<i>Listeria</i>		Prévot [3]
1966	<i>L. grayi</i>	<i>Listeria</i>		Errebo Larsen, Seeliger [9]
1971	<i>L. murrayi</i>	<i>Listeria</i>		Welshimer, Meredith [10]

Table II

Progress in the classification of *Listeria* strains during 1972-1984

Year	Species (Subspecies)	Genus	Family	References
1973*	<i>L. monocytogenes</i> group 1 group 2			Stuart, Welshimer [20]
	<i>L. grayi</i>			
	<i>L. murrayi</i>			
	<i>L. denitrificans</i>			
1974	<i>L. monocytogenes</i> serotype 5 (<i>L. bulgarica</i>)	<i>Listeria</i>		Ivanov [8, 14]
1974*	<i>L. monocytogenes</i>	<i>Listeria</i>		Stuart, Welshimer [21]
	<i>Murraya grayi</i>	<i>Murraya</i>	<i>Listeriaceae</i>	
	<i>M. murrayi</i>			
1974	<i>L. monocytogenes</i>		Genera of uncertain affiliation	Seeliger, Welshimer [7]
	<i>L. denitrificans</i>			
	<i>L. grayi</i>	<i>Listeria</i>		
	<i>L. murrayi</i>			
1979-81	<i>L. innocua</i>	<i>Listeria</i>		Seeliger, Höhne [4], Seeliger, Schoofs [12]
1981-84*	<i>L. monocytogenes</i>			
	<i>L. ivanovii</i>	<i>Listeria</i>		Rocourt, Grimont, Grimont, Seeliger [26], Rocourt, Schrettenbrunner, Seeliger [27], Rocourt, Grimont [25], Seeliger, Rocourt, Schrettenbrunner, Grimont, Jones [31]
	<i>L. innocua</i>			
	<i>L. welshimeri</i>			
	<i>L. seeligeri</i>			
	<i>L. grayi</i>			
	<i>L. murrayi</i>			

* By genetical methods

As to the future it seems very important to classify every kind of bacterium in the same way. Therefore, some points of view which have determined the classification of not only *Enterobacteriaceae* but of other bacteria also, ought to be used in case of *Listeria*.

Genetic methods were first applied by Stuart and Welshimer [20, 21] for the classification of *Listeria* strains in 1973. Their results can be seen in Table II. Rocourt and her co-workers [22-29, 31] followed Stuart and Welshimer and produced some genetic basis for the systematization of these bacteria. Their scheme is presented in Table III in a modified form. Haemolytic character

Table III

Markers for the identification of *Listeria* species (Rocourt [22])
(Modified)

	Haemolysis on horse blood agar	CAMP test with <i>S. aureus</i>	CAMP test with <i>Rhodococcus equi</i>	Production of acid from				Nitrate reduction to nitrite
				D-mannitol	L-rhamnose	D-xylose	α-methyl-D-mannoside	
<i>L. monocytogenes</i>	+	+	—	—	+	—	+	—
non-haemolytic	—	—	—	—	+	—	+	—
<i>L. innocua</i>	—	—	—	—	v	—	+	—
<i>L. seeligeri</i>	+	+	—	—	—	+	v	—
non-haemolytic	—	—	—	—	—	+	v	—
<i>L. welshimeri</i>	—	—	—	—	v	+	+	—
<i>L. ivanovii</i>	+	+	+	—	—	+	—	—
<i>L. grayi</i>	—	.	.	+	—	—	.	—
<i>L. murrayi</i>	—	.	.	+	v	—	.	+

and virulence besides acid production from sugars are in the centre of the classification of *Listeria* species. This system may have practical value. Now spontaneous disappearance of haemolytic character of *Listeria* strains is a reality, but its probability is not known. Existence of at least two non-haemolytic *L. monocytogenes* and some non-haemolytic *Listeria seeligeri* strains is a fact [32, 33]. In the case of these strains there is no possibility for the classification. Neither a non-haemolytic *L. monocytogenes* strain can be differentiated from a *Listeria innocua* strain, nor a non-haemolytic *L. seeligeri* strain can be distinguished from a *Listeria welshimeri* strain on the basis of the above mentioned characters. Serological features of these strains may serve for orientation but this character has no taxonomic importance. The ability to

Table IV

Proposal for the classification of species and biogroups of Listeriaceae [35]

Genus	Species	Biogroups	Genomic species* (groups)	Serovar (serotype)
<i>Listeria</i>	<i>L. monocytogenes</i>	1	<i>L. monocytogenes</i> (1)	1/2
		2	<i>L. seeligeri</i> (5)	
	<i>L. ivanovii</i>		<i>L. ivanovii</i> (2)	1
	<i>L. innocua</i>	1	<i>L. innocua</i> (3)	1
		2	<i>L. welshimeri</i> (4)	
<i>Murraya</i>	<i>M. grayi</i>	1	<i>M. grayi</i>	
		2	<i>M. murrayi</i>	

Results of biochemical tests should be evaluated after two days incubation. Classification of the strains into species or biogroups has to be performed by computer analysis.

* By Rocourt et al. [26]

Table V

Proposal for the classification of species and subspecies of the genus Listeria (1990)

Genus	Species	Subspecies (biotypes)	Serovar (serotype)
<i>Listeria</i>	<i>L. monocytogenes</i>	subsp. <i>monocytogenes</i>	1/2
		subsp. <i>seeligeri</i>	1/2
		subsp. <i>innocua</i>	
		subsp. <i>welshimeri</i>	
	<i>L. ivanovii</i>	—	
	<i>L. grayi</i>	subsp. <i>grayi</i>	
		subsp. <i>murrayi</i>	

lyse erythrocytes of various animal species is found in many, otherwise entirely different bacteria: streptococci, staphylococci, enteric bacteria, vibrios, corynebacteria etc., but this character does not play a key role in their classification. A group B *Streptococcus* can lose its haemolytic capacity but it still remains a group B *Streptococcus*. Haemolytic *Escherichia coli* does not belong to a new species. Virulence is certainly not a factor which determines the species. Therefore, it has been suggested that neither haemolytic property nor virulence possesses taxonomic importance in case of *Listeria* [34]. In 1985 at Nantes a scheme was proposed for the classification of *Listeria* strains [35] — see Table IV. Since then Rocourt [23] and Jones [36] summarized the problems of taxonomy of *Listeria* strain. At present it seems perhaps more adequate to include three species in the genus *Listeria* (Table V). The first species is *L.*

monocytogenes which has four subspecies or biotypes; the second *L. ivanovii* and the third *L. grayi*. The latter consists of two subspecies or biotypes *L. grayi* subsp. *grayi* and *L. grayi* subsp. *murrayi*. The recent results of Collins [37] support this view and the newest observation of King et al. [39] harmonize well with our proposal.

REFERENCES

1. Murray, E. G. D., Webb, R. A., Swann, M. B. R.: J Path Bacteriol **29**, 407 (1926).
2. Prévot, A. R.: In Dumas, J. (ed.): Bactériologie médicale. Flammarion, Paris 1957.
3. Prévot, A. T.: Traité de systématique bactérienne. Dunod, Paris 1961.
4. Seeliger, H. P. R., Höhne, K.: In Berger, T., Norris, J. R. (eds): Methods in Microbiology. Vol. 13. Academic Press, London 1979.
5. Stuart, M. R., Pease, P. E.: J Gen Microbiol **73**, 551 (1972).
6. Breed, R. S., Murray, E. G. D., Smith, N. R.: Bergey's Manual of Determinative Bacteriology. 7th ed. Williams and Wilkins, Baltimore 1957.
7. Seeliger, H. P. R., Welshimer, H. J.: In Buchanan, R. E., Gibbons, N. E. (eds): Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore 1974.
8. Ivanov, I.: Bull Off Intern Epizoot **48**, 571 (1957).
9. Errebo Larsen, H., Seeliger, H. P. R.: In Proceedings of the Third International Symposium on Listeriosis, July 13-16, 1966, Bilthoven. Rijks Instituut voor de Volksgezondheid, Utrecht 1966.
10. Welshimer, H. J., Meredith, A. L.: Int J System Bacteriol **21**, 3 (1971).
11. Ralovich, B.: Listeriosis Research; Present Situation and Perspective. Akadémiai Kiadó, Budapest 1984.
12. Seeliger, H. P. R., Schoofs, M.: In Ivanov, I. (ed.): Proceedings of the Seventh International Symposium on Listeriosis, Varna, 1977. National Agroindustrial Union Center for Scientific Information, Sofia 1979.
13. Jones, D.: J Gen Microbiol **87**, 52 (1975).
14. Ivanov, I.: Mh Vet-Med **17**, 729 (1962).
15. Sohler, R., Benazet, F., Piechaud, M.: Ann Inst Pasteur **74**, 54 (1948).
16. Pirie, J. H. H.: Nature **145**, 264 (1940).
17. Seeliger, H. P. R., Schrettenbrunner, A., Pongratz, G., Hof, H.: Zentralbl Bakteriell [A] **252**, 176 (1982).
18. Wilson, G. S., Miles, A. A.: In Topley and Wilson's Principles of Bacteriology and Immunology. Arnold, London 1955.
19. Farmer, III, J. J., Davis, B. R., Hickman-Brenner, F. W., McWhorter, A., Huntley-Carter, G. P., Asbury, M. A., Riddle, C. G., O'Hara, C. M., Morris, G. K., Smith, P. B., Brenner, D. J.: J Clin Microbiol **21**, 46 (1985).
20. Stuart, S. E., Welshimer, H. J.: Int J System Bacteriol **23**, 8 (1973).
21. Stuart, S. E., Welshimer, H. J.: Int J System Bacteriol **24**, 177 (1974).
22. Rocourt, J.: Thesis. Université de Paris-Sud, Centre D'Orsay, 1987.
23. Rocourt, J.: Acta Microbiol Hung **36**, 285 (1989).
24. Rocourt, J., Catimel, B., Schrettenbrunner, A.: Zentralbl Bakteriell [A] **259**, 317 (1985).
25. Rocourt, J., Grimont, P. A. D.: Int J System Bacteriol **23**, 866 (1983).
26. Rocourt, J., Grimont, F., Grimont, P. A. D., Seeliger, H. P. R.: Curr Microbiol **7**, 383 (1982).
27. Rocourt, J., Schrettenbrunner, A., Seeliger, H. P. R.: Ann Microbiol (Paris) A **134**, 65 (1983).
28. Rocourt, J., Seeliger, H. P. R.: Zentralbl Bakteriell [A] **259**, 317 (1985).
29. Rocourt, J., Wehmeyer, U., Stackebrandt, E.: Int J System Bacteriol **37**, 266 (1987).
30. Seeliger, H. P. R., Jones, D.: In Sneath, P. H. A., Mair, N. J., Sharpe, M. E., Holt, J. G. (eds): Bergey's Manual of Systematic Bacteriology. Vol. 2. Williams and Wilkins, Baltimore 1986.
31. Seeliger, H. P. R., Rocourt, J., Schrettenbrunner, A., Grimont, P. A. D., Jones, D.: Int J System Bacteriol **34**, 336 (1984).
32. Herendi, Á., Ralovich, B.: Acta Microbiol Hung **36**, 289 (1989).

33. Pine, L., Weaver, R. E., Carlone, G. M., Pienta, P. A., Rocourt, J., Goebel, W., Kathariou, S., Bibb, W. F., Malcolm, G. B.: *J Clin Microbiol* **25**, 2247 (1987).
34. Ralovich, B.: *Acta Microbiol Hung* **36**, 95 (1989).
35. Ralovich, B.: In Courtieu, A. L., Espaze, E. P., Reynaud, A. E. (eds): *Listeriose, Listeria, Listeriosis, 1985-86*. Université de Nantes, Nantes 1986.
36. Jones, D.: *Acta Microbiol Hung* **36**, 113 (1989).
37. Curtis, G. D. W.: Personal communication.
38. Pirie, J. H. H.: *Publ S Afr Inst Med Res* **3**, 163 (1927).
39. King, W., Raposa, S. M., Warshaw, J. E., Johnson, A. R., Lane, D., Klinger, J. D., Halbert, D. N.: In Miller, A. J., Smith, J. L., Somkuti, G. A. (eds): *Foodborne Listeriosis*. Elsevier, Amsterdam-New York-Oxford 1990.

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Acta Microbiologica Hungarica

VOLUME 38, NUMBERS 3–4, 1991

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ACTA MICROBIOL. HUNG. AMHUEF 5 38 (3–4) 161–334 (1991) HU ISSN 0231–4622

ACTA MICROBIOLOGICA HUNGARICA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

Acta Microbiologica publishes reviews and original papers on microbiological subjects in English.

Acta Microbiologica is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
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Manuscripts and editorial correspondence should be addressed to

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Acta Microbiologica Hungarica is abstracted/indexed in Abstracts of World Medicine, Biological Abstracts, Chemical Abstracts, Chemie-Information, Current Contents-Life Sciences, Excerpta Medica database (EMBASE), Index Medicus

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PRINTED IN HUNGARY

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Acta Microbiologica Hungarica 38 (3-4), pp. 161-251 (1991)

ELEVENTH CONGRESS

ORGANISED BY THE

HUNGARIAN SOCIETY FOR MICROBIOLOGY
AND BY THE FOUNDATION OF THE HUNGARIAN SOCIETY
FOR MICROBIOLOGY

BUDAPEST, AUGUST 22-24, 1991

ABSTRACTS OF PAPERS AND POSTERS

PLENARY SESSION

L. A. BABIUK

Development of viral vaccines using bovine herpesvirus-1 as a model

Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada

Bovine herpesvirus is an important pathogen of cattle but can also be used as a model for herpesvirus infection of other species including man. Using this virus as a model we have identified the proteins involved in producing humoral and cellular immunity. These include glycoprotein gI, gIII and gIV. Although all three of these glycoproteins can induce protective immunity, gIV is the most effective in this regard. These glycoproteins have been expressed in a number of different eucaryotic and procaryotic systems. Studies clearly show that the expression system used can influence the level of immunity and protection. Although Escherichia coli produced proteins are very immunogenic as measured by ELISA, their ability to produce neutralizing antibodies and protective immunity is limited. These studies clearly show that protein conformation and/or glycosylation play an important role in inducing protective immunity. Thus it is important to take this feature into consideration in vaccine design. In addition we have identified and deleted nonessential genes of the virus to produce attenuated "marked" vaccines. The use of such vaccines in aiding the eradication of the disease as well as vectors for other vaccines will be described.

H. HASHIMOTO

Drug-resistance of clinical isolates in Japan

Gunma University School of Medicine, Maebashi, Japan

More than 150 antibiotics are now available in Japan. During the last decade, appeared 54 antibiotics including 26 cepheims and 6 new quinolones. These cepheims and quinolones caused gradual increase in isolation frequency of Gram-positive bacteria, especially of Stahylococcus aureus, and caused

many hospital infections. In 1984, new quinolones first appeared in Japan as potent wide-spectrum chemotherapeutics. But paralleled to the wide use of them, resistant bacteria appeared in Serratia, S. aureus or Pseudomonas aeruginosa and rapidly increased. Mutation of DNA gyrase was common cause of quinolones-resistance in P. aeruginosa, but the change of drug-permeability was also observed. The sites of mutations, nfxA, B, C or naIB are mapped on the chromosome. In permeability mutants, several membrane proteins disappeared or newly appeared, and resistance pattern to other antibiotics were also changed. Even by selection with sub-MIC, resistant mutants develop with prolonged incubation. Ineffective use of drugs is the cause of these resistant strains.

T. WADSTRÖM

Microbial adhesion: new concepts for development of antiadhesion strategies
to combat bacterial infections

Department of Medical Microbiology, University of Lund, Lund, Sweden

Pioneer work on how (i) oral microbes colonize teeth and various oral mucosal surfaces and (ii) studies on how enterotoxigenic Escherichia coli (ETEC) colonize the small bowel of pigs and other young animals by specific surface haemagglutination like organells (fimbriae or pili) have since more than three decades encouraged scientists to investigate how microbes interact with the mucin layer and epithelial surfaces of the respiratory, gastrointestinal and urogenital tracts. We now know that the major viral bacterial, and parasitic mucosal pathogens express specific surface proteins, often named adhesins, interact with specific gluco-conjugate ligands in the mucus layers and on various epithelia of animals and man. Such receptor structures involve specific structures of mucins as well as cell surface glucoaminoglucans (or GAG's) and various glycolipids. Interestingly, a number of these microbes such as influenza virus, Mycoplasma pneumoniae, certain malaria parasites as well as ETEC colonizing animals and man use sialic acid specific lectins (haemagglutinins) as cell surface adhesins. However, a number of mucosal associated bacteria and parasite pathogens can penetrate the mucosal barrier after the initial colonization by interactions with subepithelial basal membrane and other connected tissue matrix com-

ponents such as fibronectin, various collagens, thrombospondin, vitronectin, laminin and proteoglycan structures such as heparansulphate. Present concepts of how mucosal pathogens associate with receptors in the mucin layer on epithelial cells and in submucosal tissues will be reviewed with examples of host-parasite interactions from different mucosal surfaces with special emphasis on the gastric pathogen Helicobacter pylori and the stomach mucosa. Finally, other pathogens such as staphylococci and Group A, C and G streptococci colonize in open wounds by hydrophobic surface proteins and fibronectin and collagen binding proteins will also be summarized and how this new knowledge of host-parasite receptor ligand interactions may be used to develop anti-adhesion prophylaxis such as adhesin-based vaccines anti-adhesion therapy based on the "hydrophobic principle" will also be summarized with a future perspective in such unconventional strategies to combat infections without using antibiotic and chemotherapeutic compounds.

VIRAL VACCINES

S. A. PLOTKIN

The present and future of vaccination

Pasteur Mérieux Connaught Sérums and Vaccines, Marnes-la-Coquette, France

Vaccination, defined as the induction of immunity by artificial means that is equivalent to the protection usually derived from surviving a particular infection, is almost two hundred years old. A little over one hundred years have passed since we learned how to develop vaccines in the laboratory. The result of these discoveries has been the eradication of disease, the control of epidemics or at least the reduction of morbidity on a breathtaking scale. What is the present status of vaccines and what can we hope for the future? In the past, the emphasis has been on live, attenuated organisms or entire organisms inactivated and used as suspensions. Those strategies continue to yield useful vaccines, as for example varicella vaccine, enhanced potency inactivated polio vaccine and hepatitis A vaccine. Currently, however, the predominant trends are as follows: 1. Expression of viral antigens by cloning in yeast or other vectors, as exemplified by hepatitis B vaccines. 2. Isolation and use of purified antigens as exemplified by acellular pertussis vaccine and respiratory syncytial virus vaccine. 3. Conjugation of polysaccharides to proteins in order to improve immunogenicity for infants, as exemplified by Haemophilus influenzae type b vaccines. 4. The use of living vectors, such as vaccinia, into which genes from other organisms have been inserted, as exemplified by rabies glycoprotein. 5. Combination of antigens into single injections, in order to improve the public health effectiveness of vaccination, as exemplified by measles-mumps-rubella vaccine.

In the future, we face several problems that will require considerable ingenuity to solve. They include: 1. The need to induce local immunity of sufficient duration to prevent mucosal infections of the respiratory or gastrointestinal tracts. Although moderately effective vaccines have been developed (for example) against typhoid, rotavirus and influenza, we still have far to go in this area. 2. The need to prevent sexually transmitted diseases. Although AIDS is the most famous example, we have not been suc-

cessful with older STDs. 3. The need to deliver many vaccines, in a few injections delivered early enough in life to prevent the maximum incidence of disease. This presents the problem of how to improve on the human immune system!

CH. KUNZ

The impact of a mass vaccination campaign on tick-borne encephalitis
in Austria

Institute of Virology, University of Vienna, Vienna, Austria

Tick-borne encephalitis (TBE) has been shown to occur in Vienna, Upper Austria, Lower Austria, Burgenland, Carinthia, Salzburg and the Tirol. Of all provinces only Vorarlberg appears to be free of endemic areas of TBE virus. The public health importance of TBE with almost 700 diagnosed cases, but in reality probably more than 1000 cases in some years prompted us to start a cooperative vaccine research project with J. Keppie of the Microbiological Research Establishment, Porton, Great Britain. After intensive testing of experimental vaccine batches the inactivated virus, grown in chick embryo cells, showed highly satisfactory seroconversion rates. In 1976 "Immuno" started to produce this vaccine and made it commercially available under the trade name "FSME Immun Inject". During the previous symposium held in Baden in 1979 I was able to report data which showed that the vaccine was both safe and effective. Although grave adverse effects were not encountered, the level of acceptance of the vaccine by the Austrian population was restricted initially by side effects such as headache, malaise and pyrexia. This was overcome by an improved purification procedure which resulted in a preparation that is both immunogenic and well tolerated. With this further purified vaccine a mass vaccination campaign was initiated in 1981 for the Austrian population of approximately 5 million living in endemic areas (total population 7.5 million). After a series of 3 doses the vaccine affords a rate of protection of at least 98%. Two doses are given 2 weeks to 3 months apart and the third dose 9-12 months thereafter. Booster doses are recommended at 3 years' intervals. By 1990 more than 60% of the Austrian population had been vaccinated. This led to a dramatic decline of the incidence of the disease, from 612 cases recorded in 1982 to 89 diagnosed in 1990. The efficacy of vaccination is particularly apparent in Carinthia,

where TBE is highly endemic and vaccine coverage of the population of 500 000 people exceeds 80%. From 1972 through 1976 an annual average incidence of TBE of 140 cases was recorded, while in 1990 only 12 persons were hospitalized with overt TBE. It is conceivable that with the continuation of the vaccination campaign human disease will virtually disappear in Austria within the next 5 years.

B. J. MONTAGNON, B. FANGET, H. CHALUMEAU, L. PEYRON,
J. C. VINCENT-FALQUET, R. VINAS and P. CAUDRELIER

Vero cell line to produce viral polio and rabies vaccines

Pasteur Mérieux Sérums and Vaccines, Marcy l'Etoile, France

The Vero Cell line was applied to the production of Enhanced-Inactivated Polio Vaccine (E-IPV), oral Polio Vaccine (OPV) and Rabies Vaccine (PVRV) for humans. The cell line was expanded using the cell bank systems. With a microcarrier culture technique, a large-scale production combined with an efficient purification process has been developed. An extensive study of purity and biological safety was applied to validate several Manufacturer's Working Cell Banks (MWCB). Special emphasis was laid in the absence of any virus in the cells or any trace of viral sequence included into the Vero Cell genome. Sensitive tests were applied to check that residual cellular DNA was as low as possible per each dose of vaccines. E-IPV was licensed in July 1982 in France, and since 1983 more than 60 million doses have been inoculated into children, without any side-effects. The production capacity of this E-IPV is now more than 60 million doses per year, if needed. For PVRV, the license was delivered in France in June 1985 and over 1 million doses have been injected without any problem. For OPV, the license was obtained in 1988 and a post-marketing pharmacosurvey conducted in France showed an excellent safety for more than 350 000 vaccinees. Since August 1989, more than 150 million doses have been distributed worldwide and mostly through EPI programs in connection with UNICEF and PAHO.

M. ROUMIANTZEFF and L. TEULIÈRES

Clinical and economic advantages of modern rabies vaccines

Pasteur Mérieux Sérums and Vaccines, Lyon, Paris, France

A short review of the different rabies vaccines available for human use and particularly the modern vaccines produced in cell culture will be done. The different rabies vaccines should be classified according to the cell system used to cultivate the virus: (i) Animal systems are still employed to produce the old traditional vaccines -- Semple and SMB -- which continue to be produced in several countries; (ii) Primary cell systems, particularly Hamster Kidney and Chick Embryo cells, are used; (iii) Cell lines are presently the most interesting approach for vaccine production.

The HDCV (Human Diploid Cell Vaccine), produced according to the technique first described by Wiktor et al. in 1964, which used a fixed virus, adapted and cultured on human diploid cells, was progressively adopted in all countries of Western Europe and Northern America but has slowly replaced traditional vaccines throughout the world. The PVRV (Purified Vero Rabies Vaccine), produced by using the microcarrier technique developed by Van Wezel, retained all the advantages of the HDCV while offering the possibility of a large-scale industrial production. The clinical trial results of the PVRV vaccine are presented, showing the low level of side effects, both local and general, and the high activity. This vaccine has been used successfully in pre-exposure immunization trials, and excellent results of post-exposure treatment trials are now available, with long-term follow-up. Post-exposure antirabies immunization is currently carried out according to the schedule recommended by the WHO, that includes five separate injections during the first month (on days 0, 3, 7, 14 and 30) and an optional booster dose 90 days after the first injection. Even more, a simplified 2-1-1 schedule could replace the currently recommended schedule for postexposure immunization, reducing the treatment to only 4 doses and 3 visits. All these technical and clinical improvements offer today either economical pre-exposure immunization to target populations at risk or economical and totally sure postexposure treatment to patients bitten by proven or suspected rabid animals.

É. GÖNCZÖL, W-Z. HO, A. VELPANDI, A. SRINIVASAN and S. A. PLOTKIN

Molecular interaction between human immunodeficiency virus type 1 (HIV-1)
and human cytomegalovirus (HCMV)

The Wistar Institute of Anatomy and Biology, Philadelphia, Pa, USA,
and Institut Pasteur Mérieux, Paris, France

HCMV and the HCMV-immediate early (IE) gene products are powerful transactivators of HIV and vice versa, HIV stimulates HCMV replication. Our results show that among HIV genes, the HIV-tat gene is responsible for enhancement of HCMV. Clonal lines of human rhabdomyosarcoma (RD) cells, stably transfected with the HIV-1-tat gene (RD-tat cell lines) and infected with HCMV, enhanced expression of HCMV-IE and late proteins, as compared to control RD cells. One of the RD-tat cell lines produced infectious HCMV. The RD-tat cell lines, following transfection with recombinant plasmids containing the full or truncated forms of the HCMV-IE enhancer/promoter linked to the bacterial chloramphenicol acetyltransferase (CAT) gene, exhibited an increased CAT expression, indicating a positive regulation within the -68 to +7 region of the enhancer/promoter by the tat product. A chronically HIV-1-infected human T lymphoid cell line, SUPT-1, superinfected with HCMV, expressed HCMV-IE proteins while the parental SUPT-1 cells infected with HCMV were negative.

K. BERENCSI, É. GÖNCZÖL, D. DETAISNE, R. RANDO and S. A. PLOTKIN

Human cytomegalovirus (HCMV) glycoprotein-B (gB)-specific cell-mediated
immunity in experimental animals

The Wistar Institute of Anatomy and Biology, Philadelphia, Pa, Virogenetics, Troy,
NY, NIH-NIDR, Bethesda, Md, USA
and Institut Pasteur-Mérieux, Paris, France

Identification of the HCMV proteins involved in the induction of protective immune responses is essential for the rational development of active immunization with viral subunits. Such investigations are hampered by the strict host specificity of HCMV. The use of two types of viral expression vectors, vaccinia and adenoviruses, carrying the same HCMV genes, circum-

vents the problem of host specificity and offers a new approach to evaluating cell-mediated immunity to HCMV in experimental animals. Infection of BALB/c and CBA mice with Ad-gB-induced HCMV-gB-specific helper T cells and MHC class I-restricted cytotoxic T lymphocytes. Spleen cells were responsive in in vitro lymphocyte proliferation assays to wild-type adenovirus 5 antigen and Vac-gB antigen (stimulation index = 4-30). Wild-type vaccinia antigen, however, did not induce proliferation. In in vitro cytotoxic T lymphocyte assays, splenocytes of Ad-gB-immunized mice lysed specifically Vac-gB-infected target cells (30% cytolysis at an effector:target ratio of 50:1). Ad-gB-immune effector cells of CBA mice lysed Vac-gB-infected L929 (H2^K) targets, but not Vac-gB-infected MC57 (H2^D) targets.

I. LONTAI

The quality control and use of viral vaccines in Hungary

B. Johan National Institute of Hygiene, Budapest

Quality control has always been a basic criterion for introduction and use of any vaccines in Hungary. This is especially important nowadays for viral vaccines since except for influenza no viral vaccine production exists in Hungary, thus practically all the vaccines should be imported. The comparative examination of vaccines produced by different firms is essential under such conditions. Since new techniques have been introduced for production of certain vaccines the laboratory control tests should be adapted and extended continuously. Besides laboratory tests, field trials proved very important for collection of data on efficacy and reactogenicity of different preparations to be selected for use (e.g. rubella, MMR, hepatitis B, rabies). Occurrence and persistence of antibodies elicited by the vaccinations should be controlled and if needed the vaccination schedule modified. In this respect findings indicating the need for revaccinations against measles, for reevaluation of vaccination schedule against hepatitis B and rabies may be mentioned. Studies should also be made to measure the value of different antibody tests in indication of actual humoral immune status (e.g. comparison of HI and PNR technique for measles, mouse protection and RFFIT for rabies).

G. BOGNÁR, L. KUCSERA, T. JUHÁSZ, M. S. MERZA, B. MOREIN and T. SOÓS

Potency testing of ISCOM and conventional vaccines against BHV-1

Control Institute for Veterinary Biologicals and Drugs, Budapest, Hungary,
and Sveriges Lantbruksuniversitet, Institutionen för veterinärmedicinsk mikrobiologi,
Stockholm, Sweden

Seronegative calves were vaccinated against Infectious Bovine Rhinotracheitis. Two calves received 100 µg BHV-1 ISCOM and three calves conventional inactivated oil adjuvanted vaccines with booster immunization. Serological tests and challenge experiment were performed to compare the potency of test vaccine. Clinical symptoms, body temperature changes and virus shedding were evaluated according to virus titre and duration. The ISCOM vaccinated animals shed less virus in comparison with conventional vaccines and controls.

E. D'HONDT, A. DELEM, F. DENAMUR, A. SAFARY and F. ANDRÉ

Technical and clinical development of an inactivated vaccine
against hepatitis A

SmithKline Beecham Biologicals, Rixensart, Belgium

Hepatitis A, although neither a severe illness nor one with serious sequelae, remains a frequent cause of morbidity and occasionally mortality. At present specific immunoglobulins offer the only means of treatment. There are several possible approaches to prepare an effective vaccine: a live attenuated vaccine, a killed vaccine or a subunit vaccine made by molecular biological techniques. Since the native configuration of the viral capsid protein is most important, we have chosen to develop a killed whole virus vaccine. HM175 strain virus is replicated in cell culture (MRC-5 cells) and the crude harvest is purified in order to remove cell culture constituents. The virus is inactivated with formaldehyde and adsorbed on $Al(OH)_3$. During the whole process, the preservation of important epitopes is monitored by ELISA with monoclonals. The safety of the vaccine candidate was tested in animal models. Moreover, challenge studies were conducted in chimpanzees to demonstrate the protective ability of the vaccine. In one of the studies,

passive immunization was compared to active vaccination. The clinical development of the vaccine started late '88, and since then over 20 000 volunteers received the vaccine. To examine the immune response to the vaccine, several highly sensitive assays were developed. These assays include a test for IgM, a neutralization assay and a competition ELISA to measure the antibodies directed against the protective viral epitopes. These volunteer studies were initiated to confirm the safety, to study the immunogenicity and to demonstrate protection in field conditions. The data show that the vaccine is well tolerated and that over 95% of the recipients mount a measurable antibody response after one dose of vaccine. Antibodies persist at least one year following the primary vaccination course.

T. I. TIKCHONENKO, O. I. MIROSHNICHENKO, A. S. BORISENKO,
T. I. PONOMAREVA and L. K. ERNST

Prospects of antiviral immunity in transgenic animals
with antisense RNA genes

Institute of Agricultural Biotechnology, Moscow, USSR

Successful inhibition of replication of bacterial, animal and plant viruses by antisense RNAs (asRNA) opened the possibility of creating unnatural intracellular immunity to viral infections. The key step in this approach is the development of transgenic or chimeric animals expressing asRNA genes targeted at viruses in question. At our department some of asRNA genes which inhibited effectively in vitro replication of target viruses have been transfected into rabbit early embryos. The resulting transgenic animals with asRNA targeted at adenovirus AdH5 and bovine leukaemia virus demonstrated significant increase in the resistance to viral infections on the level of isolated cell lines in vitro, as well as on the level of whole organism. Such an approach may be used in veterinary virology and animal embryogenetics for developing farm animals resistant to viral infections, whereas for medical purposes may be used the transfer of asRNA genes to the lymphocytes or bone marrow cells.

J. MÉSZÁROS, E. HORVÁTH and E. POLYÁK-NAGY

Oral immunization against Newcastle disease with the vaccines
Vitapest and Phylavac

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest,
and Control Institute for Veterinary Biologicals and Drugs, Budapest, Hungary

Oral immunization trials were conducted with a vaccine prepared from the avirulent Newcastle disease (ND) virus strain NDV-6/10. The vaccine has been registered in Hungary by the name of Vitapest and is commercially available. The oral immunizing capacity of Vitapest was tested in SPF ($n = 21$) and non-SPF ($n = 39$) cockerels susceptible to ND. The Vitapest (Phylaxia) and LaSota (Phylavac, Phylaxia) vaccines were compared for immunogenicity in cockerels ($n = 360$) having yolk-derived immunity. In the susceptible cockerels a virus titre of $10^{5.64}$ EID₅₀/bird resulted in 100% protection, while a dose of $10^{4.64}$ EID₅₀/bird still gave 75% protection. Birds with maternal immunity were immunized at 3 weeks of age and challenged at 6 and 9 weeks of age. In birds immunized via the drinking water, maternally-derived antibodies inhibited the development of immunity more than in chickens given the vaccine in aerosol form. The most favourable results were obtained if the mean HI titre of maternal antibodies was below $2.5 \log_2$ before the immunization. Groups of 3 weeks old cockerels with $\geq 10^6$ EID₅₀ virus per bird showed 40-100% protection when challenged 3 or 6 weeks postvaccination. In the majority of groups immunized with Vitapest and challenged at 6 weeks postvaccination the degree of protection was either the same as, or better than, that found upon challenge at 3 weeks. In all Phylavac-vaccinated groups but one the degree of protection tended to decline.

E. HORVÁTH, L. TEKES and E. NAGY

An enzyme-linked immunosorbent assay for evaluating immunity of chickens to Newcastle disease vaccinated with monovalent and trivalent vaccines

Control Institute for Veterinary Biologicals and Pharmaceuticals, Budapest,
and Central Veterinary Institute, Budapest, Hungary

For controlling the potency of Newcastle disease vaccines HI test and challenge have been used in Hungary. Recently several papers have been published on measuring antibodies to ND and on controlling potency of different vaccines with ELISA. For potency testing of 4 monovalent and 3 trivalent vaccines an indirect ELISA test was developed to measure antibodies. The ELISA values were compared to HI titres and survival rate. The correlation coefficient between HI titres and ELISA absorbance values was 0.7-0.85. The correlation coefficient between survival rate (per cent) and mean ELISA absorbance values (in per cent) compared to the reference positive serum was 0.85. The challenge virus dosis was 10^6 ELD₁₀₀/chicken.

T. JUHÁSZ, L. TEKES and L. KUCSERA

Application of an ELISA test in the control of IBR vaccines

Control Institute for Veterinary Biologicals and Drugs, Budapest,
and Central Veterinary Institute, Budapest, Hungary

Potency of IBR vaccines was formerly tested in our Institute by virus neutralization test performed with the sera of vaccinated calves. The minimum requirements VN titre of the sera drawn following the second inoculation should be at least 1 : 64. Nowadays the more sensitive and easily automatizable ELISA test is preferred in both screening and potency testing in several countries. In previous experiments we have already standardized an indirect ELISA test and compared the VN and ELISA titres of the sera of calves vaccinated with IBR vaccine. The correlation coefficient between the titres was 0.789. Our experiments were now aimed to set up the minimum ELISA rate and/or ELISA titre of the vaccines corresponding to the potency criteria of the vaccines. Altogether 147 calf sera were tested originating from repeated bleedings of 29 calves immunized with conventional IBR vaccines and 6 ones with IBR-ISCOR vaccine. The serological titres were assessed by statistical methods.

I. HARABACZ

Clinical studies of a new tick-borne encephalitis vaccine

Behringwerke AG, Clinical Research Immunology, W — Marburg, FRG

A highly purified, inactivated TBE virus particle vaccine (FSME-Vaccina Behring^R) was recently registered in Germany. Phase I studies showed a dose dependence of the antibody response. More than 1500 volunteers were included in phase II and III trials. Two different immunization schedules were studied: the "conventional" schedule with i.m. vaccinations on Days 0, 28 and 300, and an "abbreviated" schedule with vaccinations on Days 0, 7 and 21. In both regimen, the vaccine proved good immunogenicity and was well tolerated. Adverse events (ADEs) included headache, fever, weakness, malaise and local injection site irritation. Generally, ADEs were mild in nature and resolved within 2 days. Clearly most ADEs occurred after the first vaccination.

Antibody titres were measured after each vaccination in enzyme-linked immunosorbent assay (ELISA), haemagglutination-inhibition test (HIT) and neutralization test (NT). In HIT, seroconversion was about 50% after the first, and 99% after the second immunization. In the abbreviated schedule, geometric mean titres in ELISA were 900 on day 21, increased to more than 3000 seven days after the third vaccination and were still about 800 after 10 months. The third vaccination in the conventional schedule was followed by a steep titre increase typical for a booster reaction. It is concluded that the vaccine is safe and immunogenic, and using the abbreviated schedule, protection can be achieved within 3 weeks. Persistence of antibodies is subjected of further investigation, and recommendations for revaccination will be based on these results.

VIROLOGY

L. A. TARASSISIN

Synthetic peptides of adenovirus capsid proteins

Institute of Microbiology and Virology, Ukrainian Academy of Sciences, Kiev, Ukraina

A topography of the antigenic determinant of the adenovirus capsid proteins is studied. According to data on hydrophilicity and flexibility of the polypeptide chain of hexon and fiber of human adenovirus type 2 the predicted epitopes were determined. Peptides containing sequences of the terminal fragments and sites with maximal hydrophilicity and flexibility were synthesized. Their antigenic activity and immunogenicity were revealed. The participation of the sites determined in the formation of adenovirus capsid protein antigenic determinants is proposed.

N. VANTSAK, S. LELCHUK, M. TSIVIN and N. DYACHENKO

Mathematic model of adenovirus immunoinactivation

Institute of Microbiology and Virology, Ukrainian Academy of Sciences, Kiev, Ukraina

We attempted to describe immunoinactivation process in the first approximation with the help of mathematic model. On the base of regressive analysis methods we have obtained a mathematic model describing the participation of antibodies, interferon and complement in the neutralization of AD intensivity. Under such condition the contribution of antibodies is the highest and of the interferon is the lowest. In the limits of the used concentration of the factors the influence of each of them (interferon and complement) taken separately on the neutralization is linear since the effectiveness of antibodies influence rises with the increase of the concentration non-linearly. The highest level of effectiveness after mutual use of factors pairs is achieved at the maximum concentration (in the examined range) of each factor. Out of three pairs, the pair (antibodies -- complement) was the most effective one. By effectivity the pair (antibodies -- in-

terferon) was the next one. We should note that the given process is not critical to the level of interferon concentration: the 5-fold alteration of interferon concentration reduces the effectiveness of neutralization by 6–7%.

GY. SZÜCS and M. ÚJ

First isolation of human enteric adenoviruses 41 in Hungary

Department of Virology, Public Health Station, Pécs, Hungary

Stool specimens taken from infants and small children below 5 years of age with acute diarrhoea were collected from routine samples submitted in 1989–1990 for virus isolation. Fifty samples of a total of 122 stools previously screened for adenovirus content by Adenolex or immunofluorescence on infected coverslip-cultures using polyclonal anti-hexon serum and found positive, were selected for inoculation into Graham 293 cells. Twenty-nine adenovirus strains were successfully cultured. From 21 samplex, viruses did not grow at all or just did poorly producing insufficient quantity of DNA. Analysing the extracted viral DNA with restriction enzymes Hind III and Sma I from the 29 successfully infected cell cultures, 6 enteric and 23 non-enteric adenoviruses were revealed. Comparing the DNA profiles of the isolated enteric adenoviruses to those of prototype strains, they were characterized as type 41 and they may be DNA-variant D12 which looks the prevailing genotype nowadays in Europe. (Strains were also Proved as Ad41-subtype M3 with monoclonal sera in Dr. J. C. de Jong's laboratory, Bilthoven, Netherlands.) These isolates are the first enteric adenoviruses found in Hungary and thus our results present evidence that enteric adenoviruses, particularly Ad41, may have a significant role in the gastroenteritis of children in our country, too.

M. TAKÁCS, GY. BERENCSI, I. MEZEY, J. M. BROJNÁS, E. BARCSAY,
E. FERENCZI, I. HOLLÓS and I. DÖMÖK

Transfusion associated hepatitis caused by flavivirus other than
hepatitis C virus

B. Johan National Institute of Hygiene, Budapest, Hungary

Hepatitis C virus was shown to be a member of the flavivirus family. Tick-borne encephalitis virus and West Nile virus, members of the same family occur in Hungary, too. Serum samples from patients suffering from transfusion associated hepatitis were tested with yellow fever virus antigens for specific IgG, and IgM using immunofluorescence test. Eight hundred serum samples were tested. Yellow fever virus related IgG antibodies were found in 232 sera. In the case of 72 patients specific IgM antibodies could also be detected. The majority of the IgM positive patients undergone surgical operation and/or blood transfusion 1 to 2 months before the onset of the disease. Fiftyfour sera positive for yellow fever virus-related antibodies were tested with HCV reagents, but only 13 were found to be positive, or cross-reacting. The 20 patients with yellow fever related antibodies were controlled with tick-borne encephalitis antigens, too. Nevertheless, no measurable cross-reaction could be detected with the West Nile virus.

The hepatitis B markers also were tested in 44 sera positive for yellow fever antibodies. There was only one, which contained HBsAg, and 10 of them proved to be positive for anti-HBcAg. The results indicate, that a non-A-non-B-non-C flavivirus is also present in the Hungarian population, which can be detected on the basis of the antigenic crossreactivity with the attenuated yellow fever virus. This virus seems to be responsible for every 11th transfusion associated hepatitis examined. The presented results of HBV, HCV and yellow fever tests suggest that double infections occur frequently, thus the seropositivity with any transfusion associated hepatitis virus is representing an elevated probability of the infection with other viruses of the entity.

A. KÁTAI, M. BENKŐ, E. SZÖLLŐSY and J. MOLNÁR

Antiviral effect of tricyclic compounds

Public Health Station, Szeged, and A. Szent-Györgyi University Medical School, Szeged, Hungary

The photodynamic and antiviral effect of several dyes, as e.g. neutral red, methylene blue were earlier published. Some of the phenothiazines and dibenzazepines have shown similar effect on herpes simplex virus. On the basis of these observations it seemed to be worthwhile to study the direct antiviral effect of some representatives of the mentioned compounds on RNA and DNA viruses with and without envelope. The antiviral effect of desipramine, imipramine, promethazine, 7,8-dioxo-chlorpromazine, chlorpromazine (CPZ) and dimethyl-aminobutyl-2-chlorphenothiazine were tested. The results were compared to the effect of some well-known antiviral drugs, e.g. IDU and Acyclovir. The antiviral effects of the phenothiazines and structurally related compounds depend on the surface properties of viruses. The DNA viruses were shown to be more sensitive to the compounds examined, than the RNA viruses tested. The Mahoney strain of poliomyelitis virus was relatively sensitive to the antiviral effect of CPZ, desipramine and its hydrogen saturated derivative (DMIH). The polysubstituted phenothiazines were relatively ineffective. The antiviral effect of the compounds on the test-viruses will be discussed in detail.

L. BÁTKAI, M. BENCZUR, B. KÁLMÁN, G. PÁLFFY and A. GUSEO

Interferon production of lymphocytes in multiple sclerosis

Microbiological Research Group of the B. Johan National Institute of Hygiene, Budapest, National Institute of Haematology, Budapest, Department of Neurology, University Medical School, Pécs, and St. George Hospital, Székesfehérvár, Hungary

Interferon production of lymphocytes from Multiple Sclerosis (MS) patients compared with that of control individuals was studied. Separation of lymphocytes was carried out according to Böyum. A suspension of 5×10^6 /ml lymphocytes in Parker's medium containing 2% fetal calf serum was prepared. UV-inactivated NDV was used as inducer in a dose of $10^{6.3} \text{LD}_{50}/\mu\text{l}$. Assay of interferon: antiviral activity of lymphocytes' supernatants (after 48 h)

was carried out by a cytopathic effect inhibition assay against VSV using WI-38 cell line. In vitro production of alpha interferon by peripheral lymphocytes of MS patients proved to be about 65% lower than that of healthy controls. Studying the interferon production of lymphocytes of caucasoid and gipsy MS patients, similar results were obtained.

G. PREMECZ, A. MARKOVITS, G. BAGI, G. VAJDA and I. FÖLDES

Direct antiviral effect of interferon inducers in the absence of interferon synthesis

Microbiological Research Group of the B. Johan National Institute of Hygiene, Budapest, and
Frédéric Joliot-Curie National Research Institute for Radiobiology and Radiohygiene, Budapest,
Hungary

It was recently shown by Fan and Maniatis (EMBO J. 1989) that human gamma-interferon gene promoter contains two different virus (and poly I:C)-inducible regulatory elements. One of these elements can be activated by interferons, too. On the basis of this structure of interferon gene promoter it can be postulated that inducers of gamma-interferon should have effects even in the absence of interferon synthesis. Previous work from our laboratory (Premez et al. FEBS Lett. 1987) has shown that poly I:C really has a direct antiviral effect at the presence of actinomycin D. Inhibition of vesicular stomatitis virus (VSV) synthesis by VSV and by spike glycoprotein of VSV (Miller and Lenard, J. Cell. Biol. 1980) also confirm our assumption. On the basis of these results it can be suggested that the signal transduction pathways of viral induction of interferon and those of interferon effects are overlapping. Examining the direct antiviral activity of other interferon inducers (different alcohols, growth factors and mitogens) we obtained new data indicating that the major pathways of induction and mode of action of interferons are realized through common signal transducing mechanisms of the cell.

J. MINÁROVITS, ZS. MINÁROVITS-KORMUTA, K. FALK, I. ERNBERG,
G. KLEIN and I. FÖLDES

Host cell dependent methylation patterns of Epstein-Barr virus DNA

Microbiological Research Group of the B. Johan National Institute of Hygiene, Budapest, Hungary
and Department of Tumor Biology, Karolinska Institute, Stockholm, Sweden

We observed earlier that the EBNA 1 and LMP coding regions of the Epstein-Barr virus (EBV) genome are highly methylated in the Burkitt's lymphoma (BL) derived cell line Rael but unmethylated in a lymphoblastoid cell line (LCL) harbouring the same virus. To examine whether this is a regular phenomenon we compared the methylation patterns of selected regions (BamHI C, W, H, M, E, K and N fragments) of EBV DNA in the representative EBV carrying cell types of normal and neoplastic origin. Analysis of HpaII/MSPI cleavage patterns showed that all probed regions were highly methylated in 6 out of 6 BL biopsies but unmethylated in 4 out of 4 LCLs immortalized by the virus. EBV DNA was also highly methylated in the nude mouse pawwaged C15 nasopharyngeal carcinoma strain and partially methylated in the C18 strain. Based on differential methylation of the viral genome in latently or productively infected cells, a role for DNA methylation in the maintenance of herpesvirus latency was proposed (Desrosiers et al. 1979, Yousuffian et al. 1982). Our data shown that both highly methylated and hypomethylated EBV genomes can be maintained in a latent state in different host cells. Thus, a high level of methylation of the EBV genome may not be the only mechanism contributing to the maintenance of the latent state.

Á. GYURIS, G. VAJDA, A. PINTÉR, M. CSIK and I. FÖLDES

Establishment of a MT4 cell line continuously producing HIV-1

Microbiological Research Group, and Department of Morphology,
B. Johan National Institute of Hygiene, Budapest, Hungary

MT4 cells carrying HTLV-1 provirus in their genomes are known to grow in vitro in clusters which rapidly disintegrate after HIV-1 infection and the cells die. After HIV-1 infection of high multiplicity we succeeded to establish an MT4 cell line growing in clusters and continuously producing the

virus (MT4/HIV-1). Morphological picture and proliferation rate of these cells do not differ from non-infected control cells. Electron microscopic investigation revealed numerous virus particles indistinguishable from HIV-1 virions. The cells proved to be nearly 100% positive by indirect immunofluorescence assay using the serum of an AIDS patient. High level HIV-1 production was observed continuously for more than 2 months by reverse transcriptase microassay. H9 and MT4 cells were successfully infected with supernates of MT4/HIV-1. Depending of the infectious dose typical cytopathic changes (multinucleated giant cells) appeared in these cells 24-48 h after infection. Western blot analysis of the concentrated MT4/HIV-1 supernate showed antigens similar to those of H9/HTLV_{IIIB} supernates. The established MT4/HIV-1 cell line seems to be very suitable to produce large quantities of the virus.

J. CZEGLÉDY, R. PÓKA, GY. VERESS and L. GERGELY

Amplification of human papillomavirus type 16 transforming genes from cervical cancer biopsies and lymph nodes of Hungarian patients

Institute of Microbiology, and Department of Obstetrics and Gynecology,
University Medical School, Debrecen, Hungary

We have examined cervical cancer biopsies and pelvic lymph nodes for the presence of human papillomavirus type 16 (HPV 16) DNA in a polymerase chain reaction (PCR) using two independent primer pairs specific for the early E (E6) and early 7 (E7) open reading frames of HPV 16. The 75 cervical biopsy specimens tested were of 3 different histological degrees according to the FIGO classification (I/B, II/A, II/B). Thirtysix of them (48%) were positive in the PCR for HPV 16. In order to obtain information about the presence of HPV 16 in metastases we also analysed pelvic lymph nodes from some of the patients (we tested altogether 65 lymph nodes from 35 women). Lymph nodes originating from 19 patients whose cervical biopsies were negative seemed to lack HPV 16 sequences. In the case of women with positive cervical PCR results (altogether 16 patients) all but one (9 out of 10) lymph node metastases were positive, while 11 of the 36 histologically negative lymph nodes were also shown to contain HPV 16 DNA. This may reflect the presence of early metastatizing cells that cannot be detected with histological methods.

T. FARKAS, J. POVEZSÁN, M. DOBOS-KOVÁCS, E. SÁGHY, I. NÉMETH
and CS. N. DRÉN

Isolation of chicken anaemia virus from broiler chickens in Hungary

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest,
Veterinary University, Budapest, and Central Veterinary Institute, Budapest, Hungary

Chicken anaemia virus (CAV) was isolated from 1-6 weeks old broiler chickens originating from different breeder flocks. Total losses were estimated to be about 7-8%, and about 25% of the chickens had not reached the market weight by 7 weeks of age. The clinical disease, the gross and histological lesions were characteristics of chicken anaemia disease. Severe growth retardation, aplastic anaemia and general lymphoid organ atrophy could be induced with high (1 ml/chicken) but not with low (0.1 ml/chicken) doses of liver homogenate of dead and/or killed chickens from the field. A chloroform resistant virus smaller than 50 nm in diameter was isolated in MDCC-MSB1 cells from both field cases and experimentally inoculated chickens. The isolated virus designated as CAV-BIA showed close antigenic relationship to the reference CUX-1 strain of CAV. Antibodies to CAV were demonstrated in field sera as well as in those collected from experimentally inoculated animals. No antibodies were found against infectious bursal disease virus, reticuloendotheliosis virus, Marek's disease virus, avian adenoviruses and reoviruses.

L. EGYED and A. BARTHA

BHV-4 virus infection in foreign host species

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

Orphan virus strains belonging to BHV-4 (Movar-type herpesvirus) have often been associated with several conditions affecting cattle such as respiratory and enteric disease, reproductive disorders, lesions of the nasal and oral mucosa, etc. It was often isolated from healthy cattle. The role in inducing disease of this particular group of bovine herpesviruses is still to be elucidated. In the last decade FeCAHV (feline cell associated herpesvirus) strains were isolated from cats suffering with urolithiasis which

proved to belong to BHV-4. The route of infection of the cats was not clarified. In the last year in a Hungarian Zoo nervous symptoms and retarded growth were observed among young (3 month old) lions. All animals were killed, from the blood lymphocytes of one lion and from the spleen of an other herpesvirus strains (BHV-4) were isolated in cocultures after 4 and 3 passages, respectively. The isolate showed some pathogenicity for Balb/c mice, they were retarded in growth, after 6-8 weeks they succumbed, and some lesions in the lungs were also observed. The pathogenic role of the NBHV-4 isolate is discussed. It seems that the lions were infected by consuming raw cattle meat.

R. PYHÄLÄ, L. KINNUNEN, N. IKONEN and T. PÖYRY

Genetic variability of influenza B and A (H1N1) viruses isolated
recently in Finland

National Public Health Institute, Helsinki, Finland

Data are available which suggest that, in influenza virus evolution, a new variant of epidemic significance rarely evolves from the previous epidemic variant. Instead, separate lineages sharing a common origin seem to coexist, and the new variant more probably evolves along an accompanying pathway. Coexisting lineages have been detected, as a rule, not until the appearance of the new epidemic variant. During severe or even moderate outbreaks it may be difficult to isolate a cocirculating deviant virus which is surviving through infection chains small in number. There may be a better chance to catch such a virus under circumstances of low epidemic activity. During the epidemic season of 1989/90 influenza B viruses antigenically related to B/Victoria/2/87 circulated in Finland at a low level. In 1990/91 sporadic cases and small localized outbreaks due to B/Yamagata/-16/88-like and A/Singapore/6/86/H1N1-like viruses were recorded. Representatives of these viruses were studied for the HA1 gene sequences which were determined directly from viral RNA or from PCR-amplified products. Phylogenetic trees were compiled to illustrate the genetic variability. In addition to viruses characterized by new evolutionary changes, a conserved influenza B virus microvariant from the near past circulated in Finland in 1989/90.

J. KISS, F. D. TÓTH, A. HORVÁTH, K. RÁK, B. TELEK and A. KISS

HTLV-I-related retroviral markers in Hungarian Patients
with malignancies of T4 cell origin

Institute of Microbiology, University Medical School, Debrecen,
National Institute for Dermatology and Veneral Diseases, Budapest, and
Second Department of Medicine, University Medical School, Debrecen, Hungary

Cell and serum samples from 20 patients with T4 cell leukaemia or cutaneous T-cell lymphoma were studied for the presence of HTLV-I-related DNA sequences and antibodies recognizing HTLV-I-antigens. DNA sequences homologous to, but not identical with HTLV-I provirus were shown by Southern blot hybridization in 5 patients. One of them had adult T-cell leukaemia and four patients had cutaneous T-cell lymphoma. RNA extracted from the cell samples of these patients expressed HTLV-specific RNA as shown by dot blot hybridization with HTLV-I probe under low stringency conditions. Sera were tested by radioimmune precipitation assay for anti-HTLV subcomponent p24 and by immunofluorescence assay. The sera of patients positive for HTLV-I-related nucleic acid sequences had antibodies cross-reacting with HTLB-I p24 and HTLV-I induced cytoplasmic antigens. Our results raise the possibility that an unknown retrovirus, related to HTLV-I, may be etiologically linked to some CD4⁺ neoplasia in areas non-endemic for HTLV-I.

J. DEÁK, R. ÓNODY, B. SZABÓ and J. FÖLDES

Evaluation of rapid diagnosis methods for the herpes simplex virus 1 infections

Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary

Three technologies were adapted for the rapid diagnosis of Herpes simplex virus 1 (HSV 1) infection: (1) for ELISA, the microtiter plates were coated with HeLa cells infected with HSV 1 as antigen; (2) the immunofluorescent assay (IFA), and (3) the indirect haemagglutination (IHA) reaction. The number of the randomly investigated sera was 116. The absolute correlation of the results with the three methods was 58.1%. The ELISA was in correlation with the IFA in 7.8% and with the IHA in 12.1%. UI 116 sera 75.9% gave positive reaction, in the group of positive sera the correlation between ELISA and IHA was 98.3%, the ELISA correlated with the IFA in 95.7%.

BACTERIOLOGY

M. KONKOLY THEGE, F. ENDRÉNYI and B. BELLA

Tendencies in antibiotic resistance and consumption in Hungary

B. Johan National Institute of Hygiene, Budapest, and
National Institute of Pharmacy, Budapest, Hungary

In Hungary reliably collected data on the sensitivity of important pathogenic bacteria to the most frequently used antibiotics have been available since 1974. Gram-negative bacteria — except Escherichia coli — were antibiotic-resistant in a high percent. Acinetobacter calcoaceticus showed the highest resistance rates. Increasing number of drug-resistant Proteus mirabilis isolates was a remarkable finding. Among Gram-positives enterococci were the most resistant, and strains with high-level aminoglycoside and penicillin resistance have emerged among them. Isolation frequency of penicillin-resistant pneumococci was extremely high. Resistance rates of almost all species were higher than those in countries with well-developed health care. Data on antibiotic consumption were collected on a national scale. Relationship between antibiotic resistance and consumption in defined daily doses will be analysed.

G. SEMJÉN, H. MILCH, I. TÓTH and P. WRIGHT

Elimination of antibiotic resistance by nalidixic acid and enrofloxacin from porcine enteropathogenic Escherichia coli

Veterinary University, Budapest, and B. Johan National Institute of Hygiene, Budapest, Hungary

Elimination of R-factors by quinolones from bacteria has been reported. No study has been undertaken on enrofloxacin (En), a fluorinated quinolone developed for animal use only. For this reason, the possible effect of En as curing agent was studied by using 15 wild-type strains of Escherichia coli isolated from pigs, and the effect was compared to that of nalidixic acid (Nal). Neither En or Nal cured the single resistant strains (n = 6). En and Nal at a concentration of 0.5 MIC eliminated resistance from 7 and 5 out

of multiresistant strains, respectively. The frequency of elimination for En and Nal ranged between 1-32% and 1-51%, respectively. After eight passages in the medium containing the drug both the number of strains losing resistance and the frequency of losses were slightly increased. Modification of broth pH to alkaline (8.2) or acidic (6.0) resulted in a pronounced elimination of resistance markers. In general, a full loss of resistance was observed but segregants with partial loss were also isolated. Detailed examination revealed that these strains harboured two about 60 Md plasmids (Hly and R) which explained the failure to show plasmid elimination after curing. Whilst the elimination of plasmids seems unlikely to be of clinical importance, these findings might be of ecological significance when these drugs gain widespread use in the therapy.

P. WRIGHT and G. SEMJÉN

Emergence of resistance to nalidixic acid and enrofloxacin
in Escherichia coli strains isolated from pigs

Department of Pharmacology and Toxicology, Veterinary University, Budapest, Hungary

The first members of the quinolone carboxylic acid group (nalidixic acid, oxolinic acid) have a narrow spectrum and rapidly develop resistance. Amongst the fluorinated derivatives, enrofloxacin (En) is an extremely active broadspectrum drug developed exclusively for veterinary use. The aim of this research, was to study the emergence of resistance to nalidixic acid (Nal) and En in Escherichia coli strains isolated from pigs after serial passages in culture medium containing half the minimal inhibitory concentrations (0.5 MIC) of these drugs. During serial passages in culture media containing Nal there was a rapid appearance of resistance to Nal. Original MIC values were 1-2 mg/l, after five passages they rose to 8-128 mg/l and after ten passages they reached 32-128 mg/l. At the same time the sensitivity to En was reduced but the strains remained clinically sensitive. After five to seven passages in culture media containing En some strains exhibited reduced sensitivity while other strains were unaffected. There was multiple increase of the MIC values after the tenth passage but clinical sensitivity was maintained. Reduced sensitivity to En accompanied resistance to Nal.

A mutation rate of less than 10^{-9} was observed in the case of En whereas for Nal this rate was between 10^{-6} – 10^{-9} , which strongly correlated with the slower emergence of resistance to En. The in vitro induced resistance is stable and partly due to reduced membrane permeability.

J. MOLNÁR, K. CSURI, GY. GUNICS, H. HASSAN, Z. RÖVID, A. KHALID,
K. CSISZÁR and M. J. NAKAMURA

Instability of plasmids in some bacteria induced by xenobiotics

Institute of Microbiology, A. Szent-Györgyi University Medical School, Szeged,
Public Health Station, Salgótarján, Hungary, and
Institute of Microbiology, University of Montana, Missoula, Mont, USA

Segregation of antibiotic resistance and other plasmids was studied in various bacterial species like Pseudomonas aeruginosa, Escherichia coli, Acinetobacter calcoaceticus, Proteus vulgaris, Proteus mirabilis, Klebsiella pneumoniae, Serratia marcescens and Staphylococcus aureus in the presence of different drugs. Antibiotic resistance and bacteriocin production were eliminated with various frequencies from the different bacterial strains. Calcium antagonists and one proton pump inhibitor have shown synergistic effect with the antiplasmid action of xenobiotics. Superhelical form of plasmid DNA was more sensitive to antiplasmid compounds than open circular or linear forms.

I. ANDIRKÓ, E. SOÓS, A. BÉNYEI and F. ROZGONYI

Correlation between methicillin resistance and membrane fatty acid
composition in Staphylococcus epidermidis

Department of Microbiology, University Medical School, Debrecen, and
Department of Physical Chemistry, L. Kossuth University, Debrecen, Hungary

The fatty acid composition of membrane lipids in the methicillin resistance (MR) Staphylococcus epidermidis 8915 and the methicillin-sensitive (MS) S. epidermidis HNCMB 110012 strains was compared. The aiC15 acid was prevalent in mono-, di-, and triacylglycerols of the MS strain, while nC24

dominated in those of the MR strain, and in its neutral pigments. There was an increasing proportion of nC24 in phosphatidic acid (PA), phosphatidylglycerol, and diphosphatidylglycerol, whereas polar pigments (PP) did not contain this acid in the MR strain. In contrast, aiC15 was the main fatty acid component in all lipids fractions of the MS strain except PA and PP. The ratio of unsaturated + branched acids to saturated ones was higher in lipids of the MS strain than in those of the MR one. Thus MR membrane seemed to be less fluid and more rigid. The results support the view that mutation to methicillin resistance is accompanied by an increased permeability barrier contributing to maintain a nonspecific resistance against beta-lactam antibiotics.

C. COCITO

Antigen 60 from Mycobacterium bovis BCG.

Properties and application to the diagnosis and prognosis of tuberculosis

Microbiology and Genetics Unit, University of Louvain Medical School, Brussels, Belgium

The complex immune response elicited by mycobacterial infection results from the interaction of different bacterial components with the host immune system. Cellular immunity plays a key role in the expression of anti-tuberculous immunity: as revealed by the tuberculin skin test. The role played by humoral immunity in tuberculosis is unclear. Humoral immunity reactions may provide precious information non otherwise available. In fact, while the cellular immunity status is quite stable (positive skin test do not become negative except in case of diseases of the immune system), the level of circulating antibodies fluctuates under the cation of feedback control mechanism.

The findings reported concern three main approaches to diagnosis and prognosis of tuberculosis: (a) An A60-based ELISA procedure for serological analysis of pulmonary and extrapulmonary tuberculosis; (b) an immunoblot technique applied to cerebrospinal fluid for diagnosis of tuberculous meningitis; and (c) a cutaneous test with intradermal injection of A60, to reveal the state of anti-tuberculous immunity. The available information indicates that these procedures represent valuable assets for diagnosis and prognosis of tuberculosis. This is particularly true for the A60-ELISA, which is ex-

pected to allow a continuous monitoring of the evolution of the disease, the success of therapy, the occurrence of relapsing, and the appearance of bacterial resistance to inhibitors. Among the numerous possible applications of the A60-ELISA, a study on the Crohn's disease (a chronic enteritis of suspected mycobacterial etiology) and an epidemiological investigation on the workers' exposure to tuberculous infection are worthy of mention.

The main advantages of A60 as reagent in replacement of PPD are: (a) a single substance, instead of a heterogeneous mixture; (b) the stability of A60 preparations, contrasting with PPD instability; (c) a precise spectrophotometric measurement of A60 (in weight units), compared with the subjective evaluation of PPD (in activity units).

The present work involves the preparation of species-specific A60 epitopes (for development of a species-specific serological test for tuberculosis), the production of DNA probes (for rapid diagnosis in different forms of tuberculosis), and an investigation on the prophylactic use of A60.

H. MILCH, G. SEMJÉN, É. CZIRÓK and M. HERPAY

Characterization of Escherichia coli strains from healthy and scouring pigs by plasmid profile analysis and various typing methods

B. Johan National Institute of Hygiene, Budapest, and Veterinary University, Budapest, Hungary

Complex typing of 63 Escherichia coli strains from pigs was carried out. The strains originated from diarrhoea and healthy pigs in a farm, where postweaning diarrhoea occurred frequently. For typing of porcine E. coli strains a new phage typing scheme was elaborated. On the basis of sero-, phage-, colicin-typing and determination of plasmid profiles and different virulence markers strains derived from diarrhoeal or healthy pigs did not showed significant difference. Plasmids of size 55-78 MD were predominant among strains derived from diarrhoeal pigs. All the antibiotic resistant strains harboured R-plasmid and additionally transferability of Hly character was also proved. All but in one case strains of identical serogroups had a different plasmid profile. Plasmid profile of strains derived from pigs form the same pen showed the same plasmid profile in 33%. Transferability of LT and ST was demonstrated.

L. EMŐDY, A. LJUNGH, T. PÁL, G. SARLÓS and T. WADSTROM

Curli like filamentous surface appendages on infantile diarrhoea
Escherichia coli isolates

Institute of Medical Microbiology, University Medical School, Pécs, Hungary, and
Department of Medical Microbiology, University of Lund, Lund, Sweden

Curli like filamentous appendages have been detected on the surface of infantile diarrhoea Escherichia coli strains of various serogroups. Strains possessing this type of surface appendages are widely distributed in various geographic areas (i.e. Papua New Guinea, Gambia and Vietnam). Expression of the curli like structure is promoted by CFA agar medium or a simple solid medium containing 1% Bacto Trypton and 1.5% Bacto Agar, pH 7.2. However, it remains undetected from media used for routine fecal cultures. This surface structure mediates a tendency for bacterial aggregation, enables bacterial cells to bind to Congo red and to connective tissue proteins, and facilitates attachment to HEp-2 cells. Curli negative derivatives of the wild type strains have been shown to be impaired in all the above capabilities. Intestinal colonization might be facilitated by interactions of these surface appendages with the epithelial lining of host mucosal surfaces.

B. NAGY, T. A. CASEY, S. C. WHIPP, H. W. MOON and L. A. ARP

Age dependent adhesion of pillated enterotoxigenic Escherichia coli
to porcine small intestinal microvilli

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary, USDA,
ARS, National Animal Disease Center, and College of Veterinary Medicine, ISU, Ames, IA, USA

Electron microscopy of two porcine intestinal isolates of enterotoxigenic Escherichia coli (ETEC) derived from fatal cases of postweaning diarrhoea and lacking known colonization factors revealed that both strains produced fine (3.5 nm) and type 1 pili. Both were tested for adhesiveness to small intestinal microvilli of pigs of different ages. Neither strain adhered to isolated intestinal brush borders of newborn pigs in vitro. However, adhesion occurred when brush borders from older pigs were used. In vitro adhesion correlated with the presence of fine pili. In ligated intestinal

loops predominantly fine pili were produced in both older and newborn pigs, but adherence was significantly greater in the former. Immunoelectron microscopy on both in vitro and in vivo samples indicated the presence of fine pili between adherent bacteria and microvilli. It is suggested that adhesion by these ETEC isolates is dependent on microvillus receptors of absorptive epithelial cells that develop progressively with age and that this adhesion is mediated by fine pili that represent new adhesins.

P. KERTÉSZ, E. BENEDEK and J. BÁNÓCZY

Possible relationship between buffering capacity and salivary
microorganisms in whole saliva

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Semmelweis University Medical School, Budapest, Hungary

Paraffin stimulated whole saliva and citric acid stimulated parotid saliva were investigated in 10 subjects. Salivary flow was 8.05 ± 3.29 ml/5 min, buffering capacity 5.20 ± 2.40 , protein and Na concentrations 2.85 ± 1.40 mg/ml and 13.34 ± 1.23 mmol/l assessing the whole saliva. The same parameters for parotid saliva were 0.91 ± 0.61 ml/5 min, 3.80 ± 0.78 , 2.72 ± 0.65 mg/ml, $12.68 \pm$ mmol/l. Significant differences were found ($p < 0.001$) between whole and parotid saliva only regarding the flow and buffering capacity. The whole saliva was tested for Lactobacillus, Streptococcus mutans and Candida albicans counts also. Between S. mutans score and differences measured in the buffering capacity of whole and parotid saliva significant correlation was found using the Spearman rank correlation ($= 0.6428$, $p < 0.05$). Our results suggest that the buffering capacity of whole saliva may be influenced by the salivary bacteria.

A. BIRÓ, J. VARGA, L. ÉR, M. PAULSSON, Å. LJUNGH and F. ROZGONYI

Comparative studies on mouse virulence, hydrophobicity, and serum protein binding of staphylococci

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Public Health Station, Debrecen, Hungary,
and Department of Medical Microbiology, Lund University, Lund, Sweden

The virulence of the hydrophilic Staphylococcus epidermidis strain 3380, the autoaggregating Staphylococcus aureus strain ISP 546 and the highly hydrophobic (SAT = 0.6) Staphylococcus haemolyticus strain E2498/86 in Balb/c mice challenged by intraperitoneal injection in dextran microcarrier solution was studied in relation to bindings of fibronectin, collagen I and IV. The high binder S. haemolyticus was the most virulent ($LD_{50} = 1.4 \times 10^7$ c.f.u./g mouse) and non-binder S. epidermidis was the least virulent ($LD_{50} = 9.75 \times 10^7$ c.f.u./g mouse), while the strongest binder S. aureus showed intermediate virulence but the highest persistence rate in the organs at 10th day after the inoculation. Thus, it seemed to be possible that high adsorptive hydrophobicity measured by interaction chromatography enhanced virulence, whereas high and multiple binding capacity to serum and tissue proteins promoted organ persistence of staphylococci.

GY. NAGY, E. PAPP-FALUSI, P. KOVÁCS, T. STROJ and F. ROZGONYI

Effect of cyclophosphamide immunosuppression on coagulase-negative staphylococcus infections

Department of Microbiology and Pharmacology, University Medical School, Debrecen, and
Public Health Station, Debrecen, Hungary

Balb/c mice were given 200 mg/kg cyclophosphamide intraperitoneally (ip). Three days later, mice were infected ip with Staphylococcus epidermidis, Staphylococcus haemolyticus and Staphylococcus saprophyticus at 4 inocula ranging between 2×10^8 and 20×10^8 c.f.u./ml suspended in dextrane microcarrier solution. Controls were treated only with bacteria. Mortality rates and organ persistence of cocci were significantly increased, and more peritoneal abscesses and adhesions were developed in the mice pretreated with

cyclophosphamide than in the untreated controls, regardless of the species of Staphylococcus injected. Splenomegaly was also more pronounced indicating a probably enhanced compensatory reactivity of the immune system liberated from suppression after 13 days of cyclophosphamide administration. Our results show that cyclophosphamide treatment increases the susceptibility of mice to infection by coagulase negative staphylococci and it is also responsible for a more severe course of the diseases provoked.

M. KALAUZOV, J. PECIC, Z. JELESIC, N. LERS and S. STEFANOVIC

Transferable resistance plasmids and plasmid profile analysis
of Shigella strains

Institute of Public Health, Medical Faculty, Novi Sad, and
Institute Ruder Boskovic, Zagreb, Yugoslavia

Within the last few years analysis of plasmids is being applied very productively in the investigation of outbreaks. The similarities among plasmids emphasize the homogeneity and possible clonal origin of strains. Fifteen strains of Shigella sonnei and 9 strains of Shigella flexneri 2a isolated in two epidemics during 1989 and 1991 were tested for transfer of drug resistance. The nalidix acid resistant Escherichia coli K-12 was used as the recipient. All strains carried autotransferring resistance plasmids that coded for resistance to ampicillin, carbenicillin, streptomycin, chloramphenicol, tetracyclines and trimethoprim. Transfer frequency was very high. Molecular genetic investigations of epidemic strains of S. sonnei showed that all isolates originated from the same clone and that they had plasmid virulence of 120 MD. A 80 MD plasmid was responsible for multiple resistance, as it was transferred into the recipient strain by experimental conjugation.

É. TÓTH, M. MAGOS, I. MAYER, E. FODOR and J. FÖLDES

Characterization of the beta-lactam antibiotics resistant
Gram-negative clinical isolates

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Polyresistant Gram-negative clinical isolates were selected by the ampicillin resistance. The isolated strains, e.g. Escherichia coli, Pseudomonas, Enterobacter and Acinetobacter sp. were investigated for association of the ampicillin resistance with that of other antibiotics, the rate of production and inducibility of beta-lactamase (PBL), permeability barrier, enzyme profile of the isolates, purification and classification of the isolated PBL, the isolation and gel-electrophoretic separation of the plasmids, transfer of the PBL gene by transfection and conjugation, analysis of the genetic characters of the progeny. Association of the ampicillin resistance with those of other antibiotics was observed in the following order: Enterobacter, E. coli, Pseudomonas and Klebsiella sp. The PBL may be inducible in Pseudomonas sp. The rate of PBL production and the enzyme profile are variable among the species. The permeability barrier was the most active in the Klebsiella sp. The isolated PBL belonged in majority to the groups of TEM OXA and PSE type. The isolates had more plasmids and not all of them transfected the different recipient host cells which determine the genetic characters of the progeny.

T. FODOR

The effect of gentamicin and ciprofloxacin on Mycobacterium tuberculosis
drug concentrations attainable in urine

Korányi National Institute of Tuberculosis and Pulmonology, Budapest, Hungary

Gentamicin (100 µg/ml) inhibited the growth of more than 10^4 c.f.u. of Mycobacterium tuberculosis in Sula media. Following the inoculation of gentamicin containing suspension of bacteria on Löwenstein-Jensen media, the colonies developed later but in the same number as on the control slopes. The 340 µg/ml, or 235 µg/ml of ciprofloxacin containing Mycobacterium tuber-

culosis suspension inoculated on Löwenstein-Jensen media completely inhibited the growth of $1.5-2 \times 10^4$ c.f.u. Chlorhexidine gluconate, the agent used for decontamination of sputa in Hungary, inhibited the growth of M. tuberculosis at a rate of 75-80% on Löwenstein-Jensen media. The chlorhexidine gluconate decontamination did not influence the effect of gentamicin and ciprofloxacin on M. tuberculosis inoculated on Löwenstein-Jensen media. The experiments show that culturing urine for M. tuberculosis is not useful in patients receiving ciprofloxacin, and if possible, the administration of gentamicin should be avoided before sending samples for urine culture.

J. FÖLDES, E. NAGY, I. MAYER and J. REN

Evaluation of synergism and antagonism for the combined action of antibiotics against clinical isolates of Gram-negative multiresistant bacteria

Department of Clinical Microbiology, A. Szent-Györgyi University Medical School,
Szeged, Hungary

Synergism and antagonism of antibiotics were studied by the gel diffusion screening tests as well as the checkerboard titration and time-kill technologies. The majority of the multiresistant clinical isolates showing the phenomenons belong to Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Klebsiella and Enterobacter. Synergism was shown against the Pseudomonas strains (35) using the combination of netilmicin-cefoperazon (78%), amikacin-cefoperazon (63%) and netilmicin-ceftriaxon (83%). Tobramycin-cefoperazon and netilmicin-ceftriaxon synergy were observed most frequently in the other isolates. Antagonism was observed against the Enterobacter and Pseudomonas species with ofloxacin-tetracycline and nalidixic acid-nitrofurantoin. The isobole method is a generally applicable approach for the evaluation of the shape of dose-response curves.

GY. MOLNÁR and J. PÁNOVICS

Mycoplasmacidal activity of quinolones on genital mycoplasmas

B. Johan National Institute of Hygiene, Budapest, and
Department of Urology, Semmelweis University Medical School, Budapest, Hungary

The susceptibility of Mycoplasma hominis and Ureaplasma urealyticum reference strains and 30 clinical isolates of both species was studied to the new fluoroquinolones by agar dilution method. Ciprofloxacin, ofloxacin and pefloxacin MIC₅₀ values were 1, 1 and 2 µg/ml, respectively for M. hominis. The susceptibilities of ureaplasmas were similar to these of mycoplasmas, except for ciprofloxacin (MIC₅₀ = 8 µg/ml). While mycoplasmas were only inhibited by tetracycline at 8 µg/ml, ofloxacin exerted bactericidal activity against both organisms at a concentration four times greater than MIC₅₀. The results show that the newer quinolones may be effective in the treatment of genital mycoplasmal infections.

S. NEDELKOVICS, B. KOCSIS, J. DEÁK and J. FÖLDES

Isolation and characterization of the lipopolysaccharide from the
Salmonella minnesota mR595 strain and its serological activity in ELISA for
the diagnosis of Chlamydia trachomatis infections

Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary,
and Institute of Microbiology, University Medical School, Pécs, Hungary

Chlamydia trachomatis is a human bacterial pathogen closely related to the Gram-negative organisms. It shares a common genus specific antigen with some Gram-negative strains, e.g. Salmonella minnesota mR595. This lipopolysaccharide (LPS) was extracted, purified and analysed. The compound was gaschromatographically checked, the carbohydrate and KDO content determined. The serological activity of the LPS was proved using standard sera against S. minnesota, Shigella sonnei, Escherichia coli and C. trachomatis. The LPS is built up of lipid-A and KDO. Gaschromatography did not indicate impurities. The serological activity of the LPS was measured in indirect haemagglutination test and in ELISA. The LPS was active in both tests. The LPS antigen reacted in ELISA with the sera of patients infected with C. trachoma-

tis. The availability of the LPS-ELISA for diagnosis of chlamydial infection was tested with randomly selected sera. The results of this test were in a good correlation with those of the immunofluorescence test and culture of the samples.

K. HÁBER, J. SEGESDI, M. CSIK and I. FÖLDES

Effect of coli-phage infection on the strains of Mycobacterium phlei

Microbiological Research Group, and Department of Morphology,
B. Johan National Institute of Hygiene, Budapest, Hungary

Four strains of Mycobacterium phlei -- M. phlei, SN105, SN109 and SN113 -- were infected with coli phage lambda. The infection resulted in suspensions containing phages being able to lyse cells of different M. phlei strains. Plaques produced by phages got by lambda phage infection of M. phlei strains were heterogeneous: clear, turbid and mottled plaques were found. From these different plaques eleven phage lines were isolated and purified. According to the results of DNA analysis by restriction endonucleases, these phages proved to be identical with each other as well with mycobacteriophage Bo3 isolated by Juhász and Bönicke in 1963. These results suggest that identical prophages are present in different M. phlei strains.

I. TÓTH, M. CSIK, J. PÁSZTI, I. K. WACHSMUTH and M. L. COHEN

Two 60-megadalton plasmid-specific large molecular weight proteins with almost the same size but different localization and function in

Escherichia coli 0157:H7

B. Johan National Institute of Hygiene, Budapest, Hungary, and
Centers for Disease Control, Atlanta, GA, USA

Earlier we demonstrated the significance of the serotype specific plasmid of 60 MD (p0157) in adherence of Escherichia coli 0157:H7 and genetic derivatives to tissue cultures and the adherence was mediated by surface structures other than pili. In Western blot analysis using absorbed immun-serum we detected p0157-specific proteins of 92- and 82 kD but without adhe-

sion function and in an immunogold electron microscopy study we demonstrated that the absorbed immunserum bound to the flagella of E. coli 0157:H7 whole cells. We compared the outer membrane protein (omp) patterns of an E. coli 0157:H7 isogenic strain pair by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and demonstrated p0157-specific omp fractions in range of 94-40 kD. Recently Sherman et al. demonstrated that the 94-kD omp is an adhesin. These data clarify the mechanism of the p0157-mediated adherence in E. coli 0157:H7 to HEp-2 cells and suggest the existence of two p0157-specific proteins with almost the same size but different localization and different function.

A. SZENTMIHÁLYI, GY. MOLNÁR, G. MÁRK, A. TAKÁTS, G. RÁKÓCZY and T. WADSTRÖM

Detection of Helicobacter pylori by culture and serological methods

B. Johan National Institute of Hygiene, Budapest, Gastroenterology Unit, Árpád Hospital, Budapest, First Department of Pediatrics, Semmelweis University Medical School, Budapest, Hungary, and Department of Medical Microbiology, University of Lund, Lund, Sweden

The presence of, and antibodies to, Helicobacter pylori was investigated in 133 antrum biopsy and 162 serum specimens of 100 patients (54 males, 46 females). H. pylori was cultured from 42 samples (36 persons), whereas biopsy urease test was positive in 60 cases (45 persons). Antibodies were detected by latex agglutination and enzyme-linked immunosorbent assay: 46 (30 patients) and 94 (56 patients) specimens were positive, respectively. The methods used in this study did not show a close correlation.

A. LIPCSEY, A. SZENTMIHÁLYI and I. TÓTH

Analysis of extracellular proteins as a possible method for epidemiological typing of Pseudomonas aeruginosa

B. Johan National Institute of Hygiene, Budapest, Hungary

The traditional epidemiological typing methods like analysis of phage- and pyocin-type, antibiotic resistance pattern and plasmid profile are not accurate and reproducible enough for the epidemiological typing of Pseudomo-

nas aeruginosa. Whole cell, outer membrane and extracellular proteins of 27 antigenic type strains and 118 O11a, O11b strains isolated freshly from a perinatal intensive care unit and collected earlier were examined by the sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Protein patterns of the whole cell and the outer membrane showed a considerable homogeneity. The electrophoretic analysis of extracellular proteins in combination with serotyping proved suitable for the epidemiological fingerprinting of clinical isolates belonging to the same serogroup. In addition, this method may be useful in differentiation of various serogroups and subgroups.

A. LIPCSEY, A. SZENTMIHÁLYI, I. TÓTH and J. PÁSZTI

Comparison of Pseudomonas aeruginosa O11a,O11b and O11a,O11c serological subgroups by sodium dodecyl sulphate-polyacrylamide gel electrophoresis

B. Johan National Institute of Hygiene, Budapest, Hungary

Chemical characterization of Pseudomonas aeruginosa serogroup O11 revealed that the polysaccharides of the strains are identical but the subdivision of the O11 serogroup into two subgroups remains undetermined. The common O factor O11a is associated with the polysaccharide structure. In this study we demonstrated that neither the whole cell protein nor the outer membrane protein patterns of antigenic type strains O11a,O11b and O11a,O11c showed any difference. However, comparing the extracellular protein patterns of the two reference strains by sodium dodecyl sulphate-polyacrylamide gel electrophoresis significant differences were demonstrated.

GY. TAKÁCS and GY. NAGY

Salmonella enteritidis phage type 1 infection in broiler breeders

Institute of Animal Hygiene, Veterinary University, Budapest, Hungary

Salmonella enteritidis infection was discovered in a 5-day-old broiler parent flock. Mortality had increased to 6.1% by the 7th day of age. Cloacal swabs, caecal droppings, water and feed samples, fluffs and dead-in-the-shell embryos taken from the hatchery were investigated for the presence of

salmonella. S. enteritidis was isolated from cloaca and dead-in-the-shell embryos of three grandparent flocks and fluff taken in the hatchery. All caecal droppings were negative for S. enteritidis. One of the workers proved to be positive at salmonella control for S. enteritidis. All of the isolated strains belonged to S. enteritidis phage type 1. The rapid slide agglutination test for S. gallinarum-pullorum gave positive reactions in 10-80% with the S. enteritidis infected flocks, but S. gallinarum-pullorum could not be isolated either from the grandparents or from their progenies.

GY. MOLNÁR and GY. CORRADI

Changes in semen parameters on incubation with
Ureaplasma urealyticum serotypes

B. Johan National Institute of Hygiene, Budapest, and Department of Urology,
Semmelweis University Medical School, Budapest, Hungary

Semen from healthy individuals was artificially contaminated with Ureaplasma urealyticum. Mixtures of seminal fluid containing about 15×10^6 spermatozoa/ml and U. urealyticum with a final U. urealyticum/spermatozoid ratio ranging from 10^6 to 10^3 c.f.u. were studied after 1, 2, 4 and 24 ha incubation at 37 °C. Spermatozoon motility and endosmosis of spermatozoa (swelling test) were greatly changed, as well as the agglutination of semen. The degree of these changes was directly related to the U. urealyticum/spermatozoid ratio and contact time. There were ultrastructural morphological changes of spermatozoa observed by electron microscopy.

CS. BOGNÁR, Á. HERENDI, ZS. SENORER, F. BARON and M. GACS

Presence of lactobacilli and corynebacteria in clinical samples

B. Johan National Institute of Hygiene, Budapest, Public Health Station, Budapest,
Public Health Station, Kaposvár, and Public Health Station, Veszprém, Hungary

In consequence of the development of isolation procedures and widespread application of up-to-date medication, lactobacilli and corynebacteria are isolated in increasing numbers from clinical samples. Frequency of their

isolation raises two problems: (i) their pathogenicity is not cleared up; (ii) the variable morphology of their cells and colonies, and the biochemical properties cause difficulties in distinguishing them from streptococci and listeriae. In 1989-1990 499 Corynebacterium and 230 Lactobacillus strains were isolated from several clinical samples. Corynebacteria and lactobacilli could be differentiated from streptococci and listeria on the basis of cell morphology (coccus, coccobacillus, coryneform rods, or long rods, respectively), colonial appearance (small, grey and white, opaque colonies with white centre, small, yellow and white colonies or yellow and white colonies with large alpha-haemolytic zone, respectively), catalase and benzidine reactions and esculin-hydrolysis. Certain lactobacilli strains are resistant to vancomycin.

CS. BOGNÁR, Á. HERENDI, T. MAJOR SR. and B. BÁNKUTI

Bacteriology and antimicrobial therapy in lower respiratory tract
infections

B. Johan National Institute of Hygiene, Budapest, and Institute of Pulmonology,
Hungarian Railways, Budapest, Hungary

Bacteriological and sensitivity examination of 508 samples (sputum 339, blood culture 156, pleural fluid 11, bronchoscopic aspirate 2) deriving from 256 patients with lower respiratory tract infection was performed in a 13 months period. Microorganisms were cultured from 189 samples (37.2%); sputum samples prepared by Mulder's technique were positive in 48.4% and the blood cultures in 25%. The most frequent diseases were exacerbation of chronic bronchitis and pneumonia. Presumptive treatment and therapy based on bacteriological findings were likewise applied. Due to therapy selected on susceptibility testings, the patients became bacteriologically negative in 44.4% at the first control. In 15.3% of the patients the sputum was negative at beginning treatment. In patients with pneumonia emergency reasons usually necessitated presumptive treatment. Antimicrobial treatment in chronic bronchitis evoked a more than 50% negativity by the time of first bacteriological control examination. Advantages of antimicrobial therapy based on laboratory findings are a short duration of treatment and higher efficacy. Nevertheless, presumptive therapy with appropriate antimicrobial agents may also be successful.

J. IVÓCS, CS. BOGNÁR and Á. HERENDI

Differentiation of Listeria, Corynebacterium, Lactobacillus and Streptococcus by gas-chromatographic analysis

B. Johan National Institute of Hygiene, Budapest, Hungary

Differentiation among Listeria, Corynebacterium, Lactobacillus, Streptococcus requires more attention because these strains may show similar colonial morphology and biochemical characteristics. Cultivation and gas-chromatographic investigation of 9 Corynebacterium, 29 Lactobacillus, 23 Streptococcus, 1 Listeria strains were performed. Lactobacillus strains were microscopically highly pleomorphic. Non-volatile metabolites were analysed after cultivation in peptone yeast extract glucose (PYG) medium. HP 5720/A instrument was used. The retention times were measured and compared with those of the standard volatile acid solutions. Lactobacilli uniformly showed a single high peak of acetic acid. A high peak of acetic acid and a lower one of butyric acid was characteristic of Corynebacterium whereas streptococci were marked with a high peak of acetic acid, and a lower one of propionic acid. Propionic acid, acetic acid and iso-valeric acid were detected from the samples of the Listeria strain. Analysis of acid products by gas-chromatographic procedure seems to be suitable for differentiation of these genera.

T. T. KRAMER and M. B. ROOF

Effects of neutrophilic leukocytes on salmonellae

Department of Veterinary Microbiology, College of Veterinary Medicine, Iowa State University, Ames, IA, USA

Swine paratyphoid, due to Salmonella chlorae-suis is a septicaemic disease of swine which resembles human typhoid fever, and assumes currently epidemic proportions in the American Mid-West. We have discovered clinically healthy pigs which harbour S. chlorae-suis exclusively in blood polymorphonuclear neutrophil leukocytes (neutrophils), after separation on a Percoll gradient, and not from blood, buffy coat, or any of the 21 organs cultured on post-mortem. Occasionally salmonellae belonging to other serogroups (B

and D, in particular) were isolated from other organs and faeces. We have next adapted virulent field strains to survival in neutrophils or lysosomal granules. Neutrophil/lysosome adapted S. cholerae-suis were avirulent for mice, had a 1000x increased LD₅₀ for mice, and possessed a reduced capability to colonize the spleens of mice. The neutrophil/lysosome adapted S. cholerae-suis was also avirulent and immunogenic for pigs. Neutrophil/lysosome adapted S. chlorerae-suis invaded and multiplied slowly in neutrophils and in Vero cells, and were resistant to killing by oxidative processes by comparison with the wild type source strain. Neutrophil/lysosome adapted S. cholerae-suis have lost the 50Kd salmonella virulence plasmid. Upon re-introduction of the plasmid, virulence for mice was only partially restored. These observations suggest a peculiar adaptive milieu for the long-term survival of S. cholerae-suis in the pig, consistent with the concept of salmonellae as facultative intracellular parasites, adapted to the lymphoreticular system.

T. PÁL, P. D. CAM and A. A. LINDBERG

Inhibition of epithelial cell invasion of shigellae by human serum
and secretory antibodies

Department of Clinical Bacteriology, Karolinska Institute, Stockholm, Sweden, and
Institute of Microbiology, University Medical School, Pécs, Hungary

While there are reports indicating that the protection against dysentery is serotype specific, there are data suggesting the existence of heterologues immunity, as well. We studied in vitro a possible mechanism of this serotype (LPS) independent antishigella immunity, e.g. prevention of epithelial invasion by human antibodies specific to bacterial surface antigens. Sera from Vietnamese persons with high Invasion Plasmid coded Antigen (Ipa)-specific titers were able to inhibit invasion significantly better than samples from Swedish individuals with low titers of the same specificity. The antigens recognized by the inhibiting antibodies were shown to be LPS-independent, and were coded by the invasion plasmid. Colostral samples with high anti-Ipa sIgA titers provided similar results. Since it is known that anti-Ipa sIgA antibodies are present on the mucosal surface following natural infection or vaccination, our data indicate that prevention of epithelial invasion by antibodies could indeed be an effector mechanism of anti-dysentery immunity.

L. FODOR and Z. PÉNZES

Detection of Pasteurella haemolytica antigens by coagglutination test

Institute of Microbiology and Infectious Diseases, Veterinary University, Budapest, Hungary

A serotype specific coagglutination test has been adopted for detection of antigens of Pasteurella haemolytica in tissues of infected animals. Suspensions, saline extracts and boiled extracts made of the 16 serotype reference strains of P. haemolytica gave positive reactions with the homologous hyperimmune sera bound to a protein-A producing Staphylococcus aureus strain and only two further one-way cross-reactions (between P. haemolytica A12 serotype reference strain and anti-A2 immune serum and A13 strain and anti-A5 serum) appeared compared to the results of the passive haemagglutination test generally used for serotyping P. haemolytica. The coagglutination test proved to be capable of detecting P. haemolytica antigens in 23 out of 64 lungs of slaughtered sheep even if cultures were negative. In case of 23 experimentally infected and slaughtered or due to pneumonia succumbed calves both cultures and the coagglutination test gave positive results.

T. MAGYAR and R. B. RIMLER

Detection of toxin-producing Pasteurella multocida with a membrane assay

Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary, and
US Department of Agriculture, Agricultural Research Service, National Animal Disease Center,
Ames, Iowa, USA

Colonies of toxin-producing Pasteurella multocida were detected with peroxidase-labelled monoclonal antibodies using a colony-blot assay. Twenty-nine P. multocida isolates representing different geographic origins, hosts, capsule serogroups, and somatic serotypes were used in the study. There was a complete agreement between a filtered sonicate of P. multocida being lethal for mice and positive colony reaction in the membrane assay. No cross-reactions were observed with Bordetella bronchiseptica and Bordetella avium strains that can be associated with P. multocida in producing diseases of animals. The assay could be used to examine several isolated strains for toxin-production or enumerate toxin-producing colonies in mixed cultures.

J. FÖLDES, J. DEÁK and ZS. NEDELKOVICS

Evaluation of the different technologies for the diagnosis of
human chlamydial infections

Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary

Samples and sera of patients with clinical evidence of chlamydial infection as an aetiological agent of acute urethral syndrome and non-gonococcal urethritis or conjunctivitis were investigated. The following laboratory methods were adopted for the comparative studies: (1) ELISA using the bacterial lipopolysaccharide (ReLPS) as antigen from Salmonella typhi-murium (S. minnesota mR595) isolated with a mixture of phenol, chloroform and petroleum ether; (2) the indirect immunofluorescence test with antigen slides from bioMérieux and FITC labelled antihuman IgM and IgG; (3) the cultivation of the microorganism in tissue culture of McCoy cells following the detection of elementary bodies and inclusions by FITC labelled monoclonal antibody and in some cases by staining with propidium iodide. Of 256 random samples 72 were positive in tissue culture. The sera of patients with positive culture contained specific IgM in 16 and specific IgG in 18 cases. Twenty sera contained both IgM and IgG antibodies. The sera of patients with negative culture were positive for IgM in 14 and for IgG in 11 cases. The positive ELISA with ReLPS antigen showed a good correlation with the above-mentioned results.

V. JERANT-PATIC, J. ZIMAROV and V. VUJKOV

Chlamydia trachomatis infection in women

Institute of Public Health, Medical Faculty, Novi Sad, Yugoslavia

A random specimen of 596 women was examined by the use of the direct immunofluorescent test (DIF) for the proof of the Chlamydia trachomatis antigen in endocervical swabs (by the help of monoclonal antibodies), and with the ELISA test for the proof of IgG antibodies against C. trachomatis in sera, and the complement-fixation reaction for the proof of antibodies against the chlamydia-group antigen. The women were previously examined

anamnestically and clinically in detail. C. trachomatis infection was revealed in 30.2% of cases, with a high percentage of positive results in all age groups. These infections were more frequently proved, to a considerable extent, in women with the diagnosis of sterility than in all other examined women, and in relation to all other inflammatory processes it was most frequently diagnosed in women with uterine cervix changes (endocervicitis and erythroplasia). A high percentage of chlamydia-positive subjects 42.2% was revealed in those women that had had pathological pregnancies and births in their past medical histories. The humoral immune response to the presence of C. trachomatis infections was proven in 79.4% of cases. Of the diagnostic tests used the combination of DIF and ELISA has been shown to be the best. It has been recommended that, in the aim of successful C. trachomatis infection diagnostics, two methods be used regularly: a direct one — for the proof of the agent in patient material DIF test, isolation of a McCoy or HeLa 229 culture of Cells — and an indirect method for the proof of specific serum antibodies.

E. NAGY, P-A. MARDH and M. PETERSON

Bacterial vaginosis. Investigation of factors influencing
the ecological balance of the vagina

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Institute of Clinical Bacteriology, University of Uppsala, Uppsala, and
Department of Obstetrics and Gynecology, Country Hospital, Eskilstuna, Sweden

The presence of lactobacilli is an important factor in the maintenance of the ecological balance of the vagina. During bacterial vaginosis (BV), the decrease of lactobacilli and an overgrowth of the mixed anaerobic bacteria can be seen. Factors which may influence this flora change were investigated. The H_2O_2 production of lactobacilli was demonstrated on agar-plates containing horseradish peroxidase and tetramethylbenzidine. Antibiosis tests were carried out on Columbia agar containing 5% horse serum, at different pHs. The effect of $FeCl_3$ (0.01 mM–5 mM) on the antibiosis was also tested. Eighty percent of the H_2O_2 -producing strains were isolated from healthy persons and 75% of the H_2O_2 non-producing strains from patients with BV. H_2O_2 -producing and non-producing lactobacilli were tested in antibiosis experiments, using different anaerobic and facultative anaerobic isolates from

BV cases. Five H_2O_2 -producing and two non-producing strains of lactobacilli showed antibiosis against 4 of 12 Peptostreptococcus and 2 of 10 Mobiluncus strains. None of 15 Bacteroides, 18 Gardnerella vaginalis and 6 Peptostreptococcus strains were inhibited. A change of the pH between 5.0 and 6.5 did not influence the antibiosis.

E. NAGY, G. FRÖMAN and P-A. MARDH

Binding of fibronectin to different strains isolated from women
with and without bacterial vaginosis

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and Institute of Clinical Bacteriology, University of Uppsala, Uppsala, Sweden

Fibronectin binding of different Gram-positive and Gram-negative bacteria has been studied extensively during the past decade. The present investigation relates to the fibronectin binding of different species obtained from the vagina of women with and without signs of bacterial vaginosis (BV). Radiolabelled (^{125}I) fibronectin was incubated with a bacterial suspension for 2 h end over end, and the incubate was then centrifuged. The supernatant was aspirated off and the radioactivity associated with the pellet was measured in a gamma-counter. Out of the lactobacilli, isolated from the vagina of healthy women (39 strains) and from the vaginal discharge of women with BV (15 strains) 9 proved to bind to fibronectin at pH 7.2. The binding was specific, since an excess of unlabelled fibronectin and its amino-terminal 29-kDa fragment effectively competed for binding, whereas bovine serum albumin, human IgG and orosomucoid did not. Decrease of the pH to 4.0 increased the binding of all of the lactobacilli tested. At pH 7.2, 8 of the peptostreptococci and 2 of the bacteroides strains likewise proved to bind fibronectin, whereas the isolates of Gardnerella and Mobiluncus sp. did not.

IMMUNOLOGY

P. S. ROHEIN, L. CURTISS and L. WONG

Altered epitope expression of apoA-I causes low lecithin cholesterol acyl transferase activity in interstitial fluid

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We have reported that interstitial fluid (IF) lecithin cholesterol acyl transferase (LCAT) activity in dogs and humans is 90% lower than expected, based on the filtration characteristics of LCAT. We obtained this information using "endogenous" substrates for LCAT assay. However, using "exogenous" apoA-I liposome substrates, LCAT activity both in human and dog IF was as expected, reflecting the filtration characteristics of LCAT. These results indicate that LCAT is present in the lymph, but its esterification activity is low. To explain this apparent discrepancy, we postulated that as a result of changes of ApoA-I conformation, LCAT activating characteristics of apoA-I are altered in IF. To test this hypothesis, a monoclonal antibody (AI-11), which was directed against the LCAT activating epitope of apoA-I, was utilized. Antibody AI-11 recognized interstitial fluid apoA-I poorly, whereas a polyclonal antibody or other monoclonal antibodies against apoA-I recognized interstitial fluid apoA-I normally. We conclude that the altered expression of the apoA-I epitope is responsible for low LCAT activity in the IF.

K. MEGYERI and I. BÉLÁDI

Effect of interferon on interleukin-2 production in chicken leukocytes

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The mitogen concanavalin A (Con-A) and Con-A-coated chicken red blood cells were used for the induction of interleukin-2 (IL-2) production by peripheral blood mononuclear cells and splenocytes. For functional determination of IL-2 activity, purified and Con-A-stimulated chicken splenocytes were applied. Con-A-coated chicken red blood cells proved a better inducer

than free Con-A. On the basis of dose-response experiments the optimal concentration of Con-A was determined. Pretreatment of leukocytes with interferon before Con-A stimulation improved the IL-2 production. Different chick interferons were used for pretreatment i.e. virus, mitogen and LPS-induced leukocyte interferons and virus-induced fibroblast interferon. All interferons applied enhanced the IL-2 production of chicken leukocytes. Thus, besides its own production chick interferon reveals a priming effect on the synthesis of IL-2.

I. S. GARSSADI, I. BÉLÁDI, Y. MÁNDI, K. RÉGELY and B. TARÓDI

Modulation of the serotonin-induced inhibition of natural cytotoxicity
by interferon

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Serotonin (5-HT) at a concentration of 10^{-3} - 10^{-7} M impaired the cytotoxicity of human natural killer (NK) cells and chicken granulocytes in whole blood. 5-HT had a similar effect when separated human lymphocytes were tested, but a higher concentration was needed than for whole blood. The most pronounced inhibitory activity was exerted when 5-HT was applied at the onset of the cytotoxic assay. No effect of 5-HT on the target cells was observed. Ketanserin at a concentration of 10^{-4} - 10^{-5} M partially reversed the inhibitory effect induced by 5-HT on the cytotoxicity of human NK cells. In contrast, ketanserin did not reverse the 5-HT induced inhibition of chicken granulocytes. Ketanserin alone exerted an inhibitory effect on the cytotoxicity of chicken granulocytes. Pretreatment of the effector cells for 1 h or 18 h with human natural interferon alpha significantly reduced the inhibition of cytotoxicity by 5-HT. A similar effect was obtained when chicken granulocytes were treated with chicken leukocyte interferon before addition of the 5-HT. Thus, interferons modulate the effect of 5-HT upon the cytotoxicity of NK cells or granulocytes.

Y. MÁNDI, GY. FARKAS, S. KARÁCSONYI and I. BÉLÁDI

The role of tumour necrosis factor in sepsis following pancreatitis

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A bioassay was used to measure serum levels of tumour necrosis factor (TNF) in 15 patients with presumed sepsis following necrotizing pancreatitis or pancreatic abscess. All patients had positive cultures from abdominal fluid and abscess discharge with Gram-negative rods; 10 of the 15 patients had positive blood cultures as well. Serum samples for TNF determinations were obtained after the onset of clinical signs of sepsis, followed by daily sampling. Besides the serum TNF content measurement, the in vitro TNF production of the leukocytes was determined. The TNF-mediated cytotoxicity of the leukocytes was measured in a ^{51}Cr release assay against WEHI cells. In addition, the in vitro induction of TNF by the leukocytes was determined after stimulation with LPS. No correlation was found between the serum TNF concentration and the severity of the illness. However, the monocytes of septic patients exerted a higher cytotoxicity than that of the monocytes of normal blood donors. Not only monocytes, but also granulocytes killed the TNF-sensitive cells. Stimulation of monocytes and granulocytes with Escherichia coli LPS and with Staphylococcus aureus, led to a higher TNF production by these cells than that observed in the control group, especially at the onset of sepsis. However, in some cases the in vitro inducibility decreased before the fatal outcome of the sepsis. The results suggest that it may be more informative to determine the TNF-producing capacity of the leukocytes rather than to measure the serum TNF level in evaluating the role of TNF in septic patients.

K. RÉGELY, J. KANCSÓ, S. HUSZ, Y. MÁNDI and I. BÉLÁDI

Natural killer activity of peripheral blood lymphocytes of patients
with physical urticaria

Institute of Microbiology, and Department of Dermatology, A. Szent-Györgyi University
Medical School, Szeged, Hungary

The natural killer (NK) cell activity against K 562 target cells was investigated in a short-term ^{51}Cr release assay in patients with physical urticaria. Thirteen of the 17 patients exhibited a significantly lower cytotoxicity ($11 \pm 3.2\%$) than that of normal blood donors ($52 \pm 4.4\%$). The impairment of natural cytotoxicity was more pronounced when the NK activity was compared in the whole blood, without further separation, and all of the patient's blood exerted low cytotoxicity. Improvement of the clinical status led to an increase in natural cytotoxicity, independently of the mode of therapy. In vitro treatment of the lymphocytes with human interferon alpha resulted in a stimulation of cytotoxicity not only in the control group, but also in patients with urticaria.

ZS. SZÉNÁSI, T. PRAZNOVSZKY, ZS. OZSVÁR and K. KIRÁLY

Differentiation of the acute and chronic stages of toxoplasmosis
by Western blot analysis

Public Health Station, Szeged, Biological Center of the Hungarian Academy of Sciences, Szeged,
and Department of Infectious Diseases, Municipal Hospital of Szeged, Szeged, Hungary

The clinical diagnosis of toxoplasmosis is usually established by the serological demonstration of specific antibodies to Toxoplasma gondii. However, the IgM and/or IgG persist in high titres after seroconversion for months. Thus, the conventional serological tests fail to estimate precisely the onset of the infection. The technique of SDS-polyacrylamide gel electrophoresis and immunoblotting was applied to study the evolution of the IgG response to T. gondii antigens in the sequential sera of acutely infected patients in order to look for the differences in IgG response during the early and late stages of infection. The ~ 35 kD molecular weight antigen component elicited an IgG response early after the onset of the infection.

It was just followed by the appearance of a band in the region of 31 kD. The intensity of these bands decreased earlier than the CFT and/or ELISA titres. The fading of the 31 kD band seems to indicate the transition of the infection to the chronic stage earlier than any other methods.

L. SZTANKOV, J. EMÖD and A. FIZIL

Nonspecific protective effect of soluble glucan against
Pseudomonas aeruginosa infection

Human Institute for Serobacteriological Production and Research, Gödöllő-Budapest, Hungary

Water-Soluble Glucan (WSG) was prepared from Saccharomyces cerevisiae. The nonspecific resistance enhancing effect of WSG was investigated by virulence-tests in mice, challenged with Pseudomonas aeruginosa F5 immunotype. CFLP random-bred mice (LATI), male and female in 16-18 g of body weight were injected with graduated doses of WSG. The period of WSG treatment was varying. Other groups of mice were treated with cyclophosphamide (CY), (10 mg/ml, i.p. 0.5 ml) before/or simultaneously with the WSG injections. The treated animals were challenged with LD₅₀ of P. aeruginosa F5 immunotype. Non-treated or saline injected animals were used as control-groups in the experiments. The results of the investigations were as follows. (i) The rate of survival in WSG-treated groups of mice was significantly the highest when the animals were given repeated doses of WSG day-today. (ii) If the WSG was injected just before the P. aeruginosa challenge there was no protective effect at all. (iii) The dose of 2 mg/0.5 ml WSG resulted the best nonspecific resistance enhancing effect against P. aeruginosa infection. (iv) The mortality of animals, immunosuppressed with CY and challenged with P. aeruginosa F5-strain was almost 100%. If the mice were treated CY and WSG the mortality has been reduced by 50%. The toxicity of WSG was investigated in mice-body-weight-losing test and proved to be a non-toxic compound. Accordingly, the virulence-, and toxicity-tests the soluble glucan seems to be an effective agent against P. aeruginosa infections.

G. KISS, R. HEYNEMAN and C. BURVENICH

Effect of beta-hydroxybutyrate on release of H_2O_2 from cattle
polymorphonuclear leukocytes

Central Laboratory, Veterinary University, Budapest, Hungary, Department of Physiological Chemistry, and Department of Veterinary Physiology, State University of Gent, Gent, Belgium

Activation of neutrophil leukocytes by chemotactic or phagocytic stimuli results in a triggering of several cellular mechanisms. A striking phenomenon, called respiratory burst, comprises the generation of a cyanide-insensitive oxygen consumption with production of superoxide anions and hydrogen peroxide. The respiratory burst activity can be measured by detection of H_2O_2 following the horseradish peroxidase mediated oxidation of reduced scopoletin. Beta-hydroxybutyrate is the ketone body of greatest concentration in the blood of healthy cows (range from 3 to 10 mg per 100 ml). The aim of this study was to investigate the effect of beta-hydroxybutyrate on the respiratory burst activity of blood PMN leukocytes. Blood was collected from the vena jugularis of healthy cows and PMN leukocytes were isolated on Percoll gradient. Viability of cells was more than 90%. Measurements of H_2O_2 released from granulocytes were conducted by adding 2 μM scopoletin and 22 nM horseradish peroxidase to a cuvette containing 2 ml of a granulocyte suspension in Hanks solution pH 7.2. The cuvettes were maintained at 37 °C by a constant temperature waterbath attached to a jacketed cuvette holder in a Vitatron spectrophotofluorometer. The activating light was set at 350 nm and the intensity of the emission fluorescence was monitored at 460 nm and recorded continuously. The system was standardized in the absence of cells with known amounts of peroxide. The amounts of H_2O_2 released from the granulocyte suspensions was assayed after stimulation with phorbol myristate acetate (PMA).

Experiments established that reproducible results were obtained at granulocyte concentrations of 5×10^5 cells/ml using 100 ng PMA/ml as maximal concentration of cellular activator. Under these conditions H_2O_2 release from bovine granulocytes could be detected after a constant latency time. In a series of experiments on different cows, the latency time before onset of burst activity together with initial rates of H_2O_2 production were assayed in the presence of several concentrations and after different preincubation times with beta-hydroxybutyrate. Result revealed that no difference could

be observed in production rates of H_2O_2 after preincubation of cells with beta-hydroxybutyrate. However, it has been established that the latency time for burst activity was considerably and significantly altered after preincubation with beta-hydroxybutyrate.

MYCOLOGY

ZS. PAPP, A. OLÁH and A. SZENTIRMAI

Polyfructose production by Penicillium chrysogenum conidiosporesL. Kossuth University, Debrecen, and Biological Research Department,
Biogal Pharmaceutical Co., Debrecen, Hungary

Several industrial penicillin producing Penicillium chrysogenum strains were capable of polyfructose production. Conidia of these microorganisms produce two forms of fructans — both under aerobic and anaerobic conditions — from sucrose or raffinose sources. One of them appears as a thickening water insoluble substance attached to the surface of conidia already after 20 min of incubation. The other is a colloidal form causing the opalescence of the supernatant. Some features of fructan production by Penicillium spores (such as time course, temperature, pH, substrate concentration optimum, etc.) were characterized as well as some enzymic properties. During a period of 24 h the fructose moieties of sucrose are converted to the polymer at a ca. 10% yield at around optimal conditions.

M. SIPICZKI, B. GRALLERT and I. MIKLÓS

Sepl, a new cell division cycle gene of Schizosaccharomyces pombe var. pombeDepartment of Genetics, L. Kossuth University, Debrecen, Hungary

Cells of the fission yeast Schizosaccharomyces pombe var. pombe are usually rod shaped, grow exclusively from their ends and divide medially. A spontaneous mutant colony with rough surface was isolated, containing cells of abnormal morphology. Most cells grew stuck together in short filaments of up to 6-8 cells, suggesting a delayed separation process after cytokinesis. The defect was not due to a reduced glucanase activity in the cell wall. Interestingly, the cells within the filaments did not grow from their tips, but from various lateral regions, forming thus branches. At the branching points anucleate cells were also produced with low frequency, probably due to abnormalities in nuclear division and abnormal positioning

of the septa. The morphological deviations could be attributed to a single recessive mutation (sepl-1). A genetic analysis based on haploidization and tetrad analysis mapped the mutation on chromosome I.

G. PÉTER and T. DEÁK

False positive urease activity of Yarrowia lipolytica

Department of Microbiology and Biotechnology, University of Horticulture and Food Industry,
Budapest, Hungary

Until recently Yarrowia lipolytica has been considered a urease positive yeast species by comprehensive monographs. However, different results were obtained using the Christensen's urea agar (CUA) and the Bacto Urea Rapid Broth (URB) for testing the urease activity of several Y. lipolytica strains. The authors aim was to find the reason for this contradiction. The capability of 16 Y. lipolytica strains to decrease the pH of the aforementioned media and some modified forms of them at 25 and 37 °C was studied. The results suggest that the colour change of the indicator of CUA may well indicate a nonspecific alcalization reaction instead of urease activity in the case of Y. lipolytica strains. Consequently Y. lipolytica is a urease negative yeast species, and for testing the urease activity of yeasts the use of URB instead of CUA is recommended.

H. E. ASHOUR, E. K. NOVÁK, T. NAGY, J. ZALA, V. KARCAGI and I. VINCZE

Comparison of Cr-III binding of two yeasts

B. Johan National Institute of Hygiene, Budapest, Hungary

Because of the increasing environmental pollution with heavy metals the Cr-III tolerance and binding of Saccharomyces cerevisiae and Rhodotorula glutinis were compared in preliminary studies. Growing in Sabouraud broth (SB) R. glutinis tolerates 1 mM Cr-III completely and S. cerevisiae partially, however, 0.1 mM does not inhibit S. cerevisiae. Both of them growing in SB with 0.1 mM Cr-III binds only some ng/mg w.w. In SB pregrown R. glutinis resting cells in saline bind from 0.1 mM ca. 10-fold, increasing with

time and concentration up to 0.3 mM, while 1 mM is partially inhibitory. S. cerevisiae resting cells pregrown in SB bind in saline also more, however, with 0.3 and 1 mM an induction after ca. 60 min leading to ca. 50-fold increase. Preadaptation in SB to 0.1 mM Cr-III decreases of binding in saline of R. glutinis, while increases that of S. cerevisiae. Low pH and presence of SB decreases binding by both species. It is supposed that in both species Al-III and Fe-III do not inhibit the Cr binding in form of Cr-VI.

E. K. NOVÁK, H. E. ASHOUR, T. NAGY, L. TRÉZL and T. B. NÉMETH

Synergism in the fungicidal effect of H_2O_2 and HCHO

B. Johan National Institute of Hygiene, Budapest, and Department of Organic Chemical Technology, Technical University of Budapest, Budapest, Hungary

H_2O_2 and HCHO when used combined exerted a considerable synergistic effect, making possible to decrease the necessary concentrations of the aggressive (H_2O_2) and the toxic (HCHO) agent. With Saccharomyces cerevisiae, Rhodotorula sp. and Geotrichum candidum yeasts the addition of a non fungicidal 0.2 M H_2O_2 to non or slightly fungicidal 0.01 M HCHO gave a successfully fungicidal combination, showing a highly superior effect than that of computed on additive basis. As there is chemical evidence for the appearance of "active" HCHO at the interaction of these substances, and under the action of this system the methylation of some amino acids (e.g. lysine) and bases of nucleic acids have already been proven by chemical and genetical methods, in case of H_2O_2 — HCHO the same effect leading to lethal outcome may be supposed.

E. K. NOVÁK, ZS. HORVÁTH, L. FAZAKAS and Z. LENGYEL

Peritonitis caused by fungi during peritoneal dialysis

B. Johan National Institute of Hygiene, Budapest, Department of Nephrology, Margit Hospital, Budapest, and István Hospital, Budapest, Hungary

Sources of infection in fungal peritonitis cases during CAPD (continuous ambulatory peritoneal dialysis) and IPO (intermittent periotoneal dialysis) are of two types, originating either from the endogenous flora of the

patient (especially candidoses) or from the environment (saprobic fungi). From 34 patients suffering from peritonitis, 126 peritoneal leavage fluids were investigated for the presence of fungi. Fungi were isolated from 6 patients: Nos 1 and 2: Candida albicans; No. 3: Candida krusei; No. 4: C. albicans + Alternaria alternata; No. 5: C. albicans + Rhizopus sp.; No. 6: Acromonium strictum + Aspergillus niger. Five of the patients were successfully cured with antifungals, while patient No. 5 was lost.

I. MIKLÓS and M. SIPICZKI

Characterization and analysis of a distiller's yeast

Department of Genetics, L. Kossuth University, Debrecen, Hungary

Breeding programmes for strain improvement in industrial yeasts are usually hampered by insufficient knowledge of their life cycle and genetics. In this work we report on the characterization and genetic analysis of a distiller's yeast and its hybridization with a laboratory yeast. The distiller's yeast was found to be diploid and heterozygous for the heterothallic mating types. It formed normal asci with four spores, but their viability was rather low (39%). To learn more about meiosis and ploidy of the strain, we isolated spores, constructed hybrids and analysed the segregation. The segregation of the hybrids was abnormal, suggesting that the hybrids were prone to undergo biased meiosis and non-disjunction of chromosomes. All strains derived from spores showed a multibudded phenotype in logarithmic phase. The reason of this feature is the delayed separation of the daughter cells. It also appeared to some extent in the hybrids, indicating that it must have been semidominant.

K. JUHÁSZ and M. SIPICZKI

Novel suppressors of the pat1-301 mutation in Schizosaccharomyces pombe

Department of Genetics, L. Kossuth University, Debrecen, Hungary

The pat1-301 gene in Schizosaccharomyces pombe is a negative regulator of the sexual pathway (both conjugation and meiosis). Strains with pat1 mu-

tation stop vegetative growth and sporulate even in the presence of a nitrogen source and mating-type homozygosity at restrictive temperature. Suppressor mutants of patl-301 mutation were isolated: mei2 (Iino-Yamamoto, 1986) and affl (Sipiczki, 1988). Besides these we isolated three new strains which are patl-301⁻ genetically, but they do not have a pat⁻ phenotype. (1) The first strain contains a suppressor mutation of patl⁻ that causes partial sterility. (2) The second strain contains two mutations, one is a suppressor of patl⁻ which is probably mei2, and the other mutation reduces sexual activity. (3) The third strain turned out to be defective in two genes, too. One of the mutations confers sterility which is not allelic with affl and the other one causes spore lethality.

Z. BENKŐ and M. SIPICZKI

Adaptability and resistance of Schizosaccharomyces pombe var. pombe
to caffeine

Department of Genetics, L. Kossuth University, Debrecen, Hungary

Stable mutants showing increased resistance to growth inhibitions by caffeine were isolated from Schizosaccharomyces pombe var. pombe after UV-irradiation of vegetative cells. Genetic analysis revealed that they comprised at least four complementation groups. Most of the mutants show rather complex phenotypes, suggesting that the products of the genes defective in them must participate in various processes and pathways. A frequent aberration is the reduced sporulation efficiency: 90% of the mutants are either impaired in sporulation or sterile. Wild type cells are inhibited by caffeine concentrations higher than 16 mM. However, by a slow step-wise increasing of the concentration of caffeine in liquid media, the cells adapt themselves to the drug and can grow even in the presence of 26 mM caffeine. The resistance acquired in this way is not stable, after a few divisions in caffeine-free medium the cells become sensitive. The mutant cafl-21 does not show this adaptation, the level of its resistance can not be increased. Mutants belonging to the other three complementation groups are now under investigation.

A. MARÁZ and L. LAKATOS

Role of mitochondria in the expression of killer phenotype
in Candida glabrataDepartment of Microbiology and Biotechnology, University of Horticulture and Food Industry,
Budapest, Hungary

Killer phenotype in some Candida glabrata strains is in accordance with the production of an extracellular glycoprotein toxin: PEST, which acts on the cell membrane of the sensitive strains causing apparent cell lysis of the attacked cells (Bussey and Skipper, 1975). Localization of the killer and immunity determinants within the nuclear and cytoplasmic genomes is not specified yet. Our attempts for the isolation of cytoplasmic RNA or DNA killer plasmids were also unsuccessful. Mutagenic action of different mutagens on the killer phenotype was studied and attempts were made for the isolation of non-killer (kil^-) mutants. Ethidium bromide was highly active in the generation of kil^- mutants which proved to be mitochondrial respiration deficient (ρ^-) ones at the same time. These mutants still maintained their immunity against the killing action of the toxin thus belonging to the category of neutral phenotype. Manganese-ions which are potent inducers of mitochondrial point mutations in the majority of yeast species were also able to generate kil^- mutants — but opposite to the formers — these mutants mostly exhibited the normal, respiratory competent (ρ^+) phenotype and all but one lost the immunity against the toxin.

Inhibitory action of the antibiotics and drugs oligomycin, mucidin and diuron was monitored on the killer toxin production. These agents cause complete inhibition of mitochondrial functions, thereby contraining the cells to metabolize glucose via the fermentation pathway. All the tested inhibitors caused complete inhibition of the killer toxin production, too. Inheritance of kil^- mutations was studied by somatic hybridization of protoplasts.

CS. FEKETE, Á. SZÉCSI and L. HORNOK

Preparation of mitochondrial DNA from Fusarium species

Agricultural Biotechnology Center, Gödöllő, and
Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary

Fusarium species belonging to the sections Sporotrichiella and Arthrosporiella are known for their ability to produce harmful trichothecene mycotoxins. There are great taxonomical controversies over these groups of fungi. The morphological classification presents many uncertainties not successfully resolved by chemotaxonomical comparison of toxin production patterns of the different species. Since restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA are highly conserved among strains but are strikingly different between species we started a program to compare mtDNA RFLPs and toxin production patterns of these fungi. The prerequisite of these experiments is a purified mitochondrion preparation free of polysaccharides and other macromolecular contaminants. A method was elaborated which produced highly purified DNA preparations suitable for cleavage by a variety of restriction endonucleases. The procedure consists of the following steps: growing young mycelium pellet in shaken cultures on nutrient-rich medium; pretreatment with 2-mercapto-ethanol; cell wall disruption by glass beads; mitochondrion precipitation by gradient centrifugation; digestion of nuclear DNA by DNAase; lysis of mitochondrion membranes; protein and polysaccharide decontamination.

A. ANDOR, A. JEKKE and Á. HÜLBER

Protoplast fusion in cyclosporin producing Tolypocladium varium strains

Institute for Drug Research, Budapest, Hungary

Genetic recombination potentially is a much more powerful method for developing strains than selection or mutation. Protoplast fusion is a possibility to produce heterokaryons or diploids and may provide a means of genetically manipulating strains of cyclosporin A producing Tolypocladium varium spec. nov. CY-93. High yields (10^8 /ml) of protoplasts can be obtained from mycelium of this strain by digestion of cell wall with Helicase and beta-

glucuronidase (1% w/v) enzyme mixture. Protoplasts were fused by addition of polyethylene glycol (PEG) and regenerated readily. We attempted to carry out genetic recombination of protoplast fusion using arginine and adenine-requiring mutants of T. varium strain. The frequency of prototroph formation was 0.1%.

K. KOROM, A. FRANKÓ, CS. VÁGVÖLGYI and L. FERENCZY

Optimisation of electrofusion for protoplasts by Aspergillus nidulans

Department of Microbiology, A. József University, Szeged, Hungary

The construction of hybrids via electrofusion of fungal protoplasts has increasing importance both in fundamental and applied research. Two haploid auxotrophic mutant strains (SZMC 1156: y, PABA⁻ ade⁻; SZMC 1157: y, PABA⁻, lys⁻) of Aspergillus nidulans were used to obtain nutritionally complementing hybrids via electrofusion. The experiments were carried out with an electrofusion instrument constructed at the Department of Microbiology. The optimized parameters were as follows: alignment field frequency, pulse voltage and pulse duration. For these two strains the highest complementation values were detected at 1.0 MHz alignment frequency, 55 μ s pulse length and 1000 V pulse voltage. Similar optimisation was also performed on different size-classes of protoplasts separated from the heterogenous original protoplast population.

J. VARGA and CS. VÁGVÖLGYI

Isoenzyme analyses of some mesophyl Mucor strains

Department of Microbiology, A. József University, Szeged, Hungary

The biological and industrial importance of filamentous fungi belonging to the genus Mucor is rapidly increasing. Thirty Mucor strains representing 10 species (Mucor albo-ater, Mucor circinelloides, Mucor corticolus, Mucor fragilis, Mucor hiemalis, Mucor mucedo, Mucor plumbeus, Mucor racemosus, Mucor ramannianus, Mucor rouxii) were compared by isoenzyme analysis.

Preparation of crude protein extracts, polyacrylamide gel electrophoresis and detection of enzyme activities on gels were carried out according to the standard methodology. Acid and alkaline phosphatases, alpha- and beta-arylesterases, catalase, glucose-6-phosphate dehydrogenase, glutamine dehydrogenase, lactate dehydrogenase, leucine aminopeptidase, NAD- and NADP-dependent malate dehydrogenases, succinate dehydrogenase, and superoxide dismutase isoenzyme patterns were determined. The results obtained were compared to the recent taxonomic classification of this genus.

J. VARGA and E. RINYU

Characterization of Aspergillus fumigatus strains by isoenzyme analysis

Department of Microbiology, A. József University, Szeged, Hungary

Aspergillus fumigatus strains are the most frequently reported agents of opportunistic fungal infections caused by the filamentous fungi. We have compared more than twenty clinical and veterinary isolates and collection strains of this species by isoenzyme analysis. Crude protein extracts were prepared from lyophilized mycelial powder of A. fumigatus strains. Polyacrylamide gel electrophoresis and detection of enzyme activities on gels were carried out according to the recent literature. Acid phosphatase, arylesterase, catalase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase, NAD- and NADP-dependent malate dehydrogenases, and superoxide dismutase isoenzyme patterns were analyzed. Intraspecific variabilities were observed in the case of the beta-arylesterase and acid phosphatase patterns. These isoenzyme polymorphisms could be useful in typing A. fumigatus strains.

B. ZARÁND and K. RÉCZEY

Operational stability of beta-glucosidase from Aspergillus phoenicis QM 329

Technical University, Budapest, Hungary

Beta-glucosidase is one of the essential enzymes in the enzymatic conversion of cellulose. This enzyme converts the intermediate cellobiose into glucose. Aspergillus phoenicis QM 329 was found to grow in shape of beads in

shake flasks and in an air-lift fermentor. The beta-glucosidase activity was retained in the beads. For inoculation, conidia were suspended in sterile distilled water containing Triton X-100. The best results were obtained when the Triton X-100 concentration was 0.8%. The beads used were approximately uniform, 2 mm in diameter. The operational stability of different kind of beads was studied with batch hydrolysis of cellobiose at 50 °C and pH 4.8. Beads size, pH profile during the fermentation, age of the beads and Triton X-100 had no significant influence on the stability. We found that the beads could be air-dried and do not lose their activity, but the operational stability of dried beads was very bad. A new method with sonication of the beads was worked out for determination of the enzyme retaintment in the mycelial pellets.

J. SERFŐZŐ and K. HALMY

Inhibitory effects of imidazole and triazole type antimycetics
on the different developmental form of Candida albicans

Institute of Biology, L. Kossuth University, Debrecen,
Department of Dermato-Venerology, County Hospital, Debrecen, Hungary

In this work the antifungal effects of ketoconazole (an inidazole derivative), itraconazole and fluconazole (triazole ones) on the formation of the different developmental forms as chlamydo-spores, mycelia, pseudomycelia and blastospores of Candida albicans have been investigated. The C. albicans strain originatig from a vaginal mycotic patient was placed on Reith rice-agar culture medium for experiments containing drugs in 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} M.ml⁻¹ concentration. The incubation period was 24, 48 and 72 h at 26 °C. The main symptoms of drug inhibitory effect appeared at the end of 24 h incubation. The antimycotic effects of the above-mentioned drugs are expressed, particularly, in the inhibition of chlamydo-spore development. The growth and differentiation of mycelia and pseudomycelia, however, are less responsive to antimycetics in question. The formation of blastospores are not inhibited during the in vitro experiments though their structural and functional damage begins at high (10^{-5} M.ml⁻¹) drug doses. On the basis of our light microscopic studies the antifungal effectiveness of the examined drugs can be ranked in the following order: itraconazole > ketaconazole > fluconazole.

J. SZABÓ, A. KARUCZKA and P. SUTKA

Serological experiments with Candida guilliermondii antigen
in psoriasis vulgaris

Town Council Hospital, Karcag, and
Institute for Animal Husbandry and Nutriment Qualification, Budapest, Hungary

Agglutination, precipitation and ELISA were applied to examine the antibody reaction of serum against Candida guilliermondii antigen in 110 patients suffering from psoriasis vulgaris. The results were compared with the frequency of C. guilliermondii positivity in healthy donors and in patients with haematologic disease. C. guilliermondii positivity is about twice higher in psoriasis vulgaris than in the healthy population. The seropositive Patients became symptom-free after systematic antimycotic therapy. C. guilliermondii might play a part in the development of psoriasis vulgaris.

AGRICULTURAL AND FOOD MICROBIOLOGY

B. M. LUND

The control of Listeria monocytogenes in food

Agricultural and Food Research Council Institute of Food Research, Norwich Laboratory,
Norwich Science Park, Colney, Norwich, UK

Recent surveys have shown that Listeria monocytogenes can be present in a wide range of foods. Measures to eliminate it from foods will be discussed. It is difficult to ensure that foods are not contaminated with this bacterium. Because L. monocytogenes can multiply at refrigeration temperatures, it is advisable that further barriers, in addition to storage at chill temperatures, are used to prevent its multiplication in foods. Experiments to determine the effects of preservative factors on the survival and multiplication of L. monocytogenes have been reported.

B. LENKEY, I. GYÜRE and A. SZENTIRMAI

Rapid detection of the steroid-11-dehydrogenase in a "sandwich" assay

Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, Hungary

Bioconversion of steroids by microorganism has great importance in the evaluation of a new derivative, a new medicine. It is a roundabout procedure to select an organism which can be able to transform any organic compounds. When the organic compound is very poorly soluble in water, it causes further problems. In our study the basic point was the solubility of steroids as substrates: 4,9(11)-androstadien-3,17-dione; 4-androsten-3,17-dione (androstenedion) and 4-pregnen-11beta,17alpha,21-triol-3,20-dion (hydrocortisone). The suitable concentration of substrate solution was prepared in a reasonable organic solvent and sprayed homogeneously onto the hydrofil membrane. After drying and sterilization, the membrane was immersed into the 50 °C Johnson agar medium and put onto the same surface of the agar. This is the so-called "sandwich" plate what was inoculated with the microorganism and incubated for a few days at 37 °C. After the incubation the membrane was

taken out of the "sandwich" plate, the parts of the agar were removed from the membrane and it was dried. When the microorganism has steroid-1-dehydrogenase enzyme it is able to transform the substrate on the membrane. The substrates can be distinguished from their delta-1-derivatives by a saturated aqueous solution of sulphosalicylic acid reagent, because they produce different colour reactions. The membrane from the "sandwich" plate was sprayed with this reagent and heated at 110 °C for 5 min. In this way we were capable of detecting as little as 0.5 µg amounts of steroids.

D. BÁNÁTI, J. FARKAS and É. ANDRÁSSY

Extension of shelf-life of refrigerated meat products by combined application of irradiation and other anti-microbial factors

Department of Refrigeration and Livestock Products Technology,
University of Horticulture and Food Industry, Budapest, Hungary

Effects of vacuum-packaging, application of ascorbic acid or an acidic sodium pyrophosphate which reduced the pH from 6.0 to 5.4, and their combination with 1 kGy dose of gamma irradiation have been investigated on the shelf-life of minced pork meat refrigerated at 2 °C. Reduction of pH resulted in a 2-6-day increase of shelf-life. Radiation treatment alone or in combination with vacuum-packaging provided a 4-9-day extension of the shelf-life. Combination of pH-reduction with irradiation did not improve further the storage stability of minced meat, due to non-microbiological quality degradation over a 3-week storage period. The shelf-life of vacuum-packaged meat rolls prepared from beef, cereal fillings and spices amounted 12 days at 0 to +2 °C, which could be increased to 30 days by 2 kGy or by reduction of the water activity with glycine to $a_w = 0.945$.

B. BIRÓ and M. KECSKÉS

Salt and pH tolerance of Rhizobium from Coronilla varia

Research Institute for Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences, Budapest, and Department of Microbiology, Agricultural University, Gödöllő, Hungary

The growth of rhizobia may be limited by soil acidity and salinity. Many authors realized these detrimental effects on the survival and growth of Rhizobium in the environment. The growth of 15 Rhizobium strains isolated from crownvetch (Coronilla varia L.) was investigated at different NaCl concentrations (1-4%). The survival of the strains in acid media was also studied at low pH (5.0-5.5) in Soerensen phosphate buffer medium.

The Rhizobium strains exhibited an improved growth at < 1% NaCl, or there were no significant differences up to 1%. They grew poorer at high salt concentration (above 2%). The population density decreased to 50% at pH 5, but there were tolerant strains, too.

M. TODOROVIĆ, V. MOLNÁR and L. JOSIMOVIĆ

Sterilization of fat-free and full-fat soya flour with ionizing radiation

Faculty of Technology, Novi Sad, Soyaprotein Industry Complex, Bečej, and B. Kidrič Institute of Nuclear Science, Beograd, Yugoslavia

The possibility of fat-free and full-fat soya flour sterilization with ionizing radiation was tested. Different kinds of flour are very often contaminated by Clostridium species and molds. As some varieties of soya flour constitute bread as the main ingredient, it was interesting to examine the effect of ionizing irradiation, which proved to be the most efficient method of the powdered food sterilization. Two sorts of flour were selected: (i) MTF (UTB) or moderately toasted flour with 2% of fat; (ii) FEAF (PEAB) or fullfatenzymic active flour with 19% of fat. These flours were deliberately contaminated by spores of Clostridium sporogenes and sterilized with doses from 5 kGy to 7 kGy.

J. BECZNER and I. F. KISS

Determination of optimum of combined treatments using
mathematical modelling

Central Food Research Institute, Budapest, Hungary

The effect of pH (4.5, 6.0, 7.5), irradiation (0, 3, 6 kGy) and heat treatment (95, 105 and 115 °C) on spores of Clostridium sporogenes (strains V 1240.2.1.1. and PA 3679) were studied. In both cases the heat resistance significantly increased in the combination of irradiation and low pH. Spores of strain PA 3679 were affected already at milder treatments (pH 4.5, 3 kGy and 105 °C), while spores of strain V 1240.2.1.1. required more aggressive treatments (pH 4.5, 6 kGy and 115 °C) to be effective. By the use of mathematical modelling the number of experiments can be reduced, and in doing so time is spared.

Z. MIJAČEVIĆ, L. STOJANOVIĆ and D. IVANOVIĆ

The activity of milk acid mesophilic bacteria in milk

Veterinary Faculty, Beograd, and Agroekonomik Co., Padinska Skela, Yugoslavia

The aim of our investigation was to find out the activity of some milk acid mesophilic bacteria in milk with different number of cells. The number of cells was the parameter by which the disturbed milk gland secretion was determined, and also the chemical structure of milk, depended on that parameter. Milk with 2×10^5 and 7×10^5 cells/ml was chosen and then 7 types of Streptococcus lactis, 4 types of S. lactis subsp. cremoris and 2 types of S. lactis subsp. diacetylactis were planted in it. The activity of chosen milk acid bacteria types was determined by measuring °SH and pH at 30 °C during 6 h incubation. The results have shown that the activity of some milk acid bacteria types were different in milk with different number of cells.

M. W. PECK, D. A. FAIRBAIRN and B. M. LUND

The effect of lysozyme on growth from heat-damaged spores of
non-proteolytic Clostridium botulinum

Agricultural and Food Research Council Institute of Food Research, Norwich Laboratory,
Norwich Science Park, Colney, Norwich, UK

Non-proteolytic strains of Clostridium botulinum can multiply at temperatures down to 3.3 °C. The trend towards the production of refrigerated, processed foods with an extended shelf-life has resulted in a need for information regarding the heat resistance of spores of these strains. Heat treatment at 85 °C causes sub-lethal damage to the spores, which remain viable but dormant, the addition of lysozyme to the recovery medium enables germination and growth, in a study of four strains of type B and two of type E the addition of 5–10 µg (312–625) lysozyme/ml resulted in detection of the maximum number of surviving spores of most strains. Spore suspensions were found to contain both heat-sensitive and heat-resistant spores which formed approximately 99.9% and 0.1%, respectively, of the total spores. Growth of these strains was not inhibited by lysozyme concentrations of up to 100 µg/ml, this contrasts with the sensitivity to lysozyme shown by Clostridium tyrobutyricum. Lysozyme is present in some foods and is relatively heat-resistant; it may enable growth from sublethally damaged spores of non-proteolytic C. botulinum in some mildly heat-treated, chilled foods.

A. H. DAWOOD, S. M. ABD-ELHADY, S. M. ABDOU and A. M. ABD-ELAATY

Production and some properties of beta-galactosidase from fungi

Food Science Department, Faculty of Agriculture, Zagazig University, Moshtoher

Beta-galactosidase is an enzyme which can be produced by several organisms i.e. bacteria, yeasts and fungi. Although some bacteria exhibit high lactase activity, they produce intracellular enzyme and relatively low cell yields, but fungi produce higher cell yields. Aspergillus oryzae DSM 1864 was selected from 18 tested fungi strains as the most active organism for beta-galactosidase production. This was achieved after growing on wheat bran semi-solid medium adjusted to pH 6.0–6.5, inoculated with active spores sus-

pension and incubated at 30 °C for 72 h. Acetone precipitation method revealed the highest recovery (20%) of beta-galactosidase as compared with the precipitation by saturated 100% ammonium sulphate (57.5%). The storage stability of A. oryzae DSM 1864 beta-galactosidase precipitated with acetone was 12 days at room temperature 25 °C, 63 days in refrigerator 6 °C and 126 days under freezing -10 °C. A sudden decrease in the enzyme activity was observed when the storage time under freezing was prolonged over 126 days.

H. E. A. F. BAYOUMI and M. KECSKÉS

Effect of combined inoculation of Pseudomonas fluorescens with Rhizobium strains on nodulation and dry matter of broad bean in the presence of ammonium nitrate

Department of Microbiology, Agricultural University, Gödöllő, Hungary

Laboratory and glass-house experiments were carried out to determine the effect of Pseudomonas fluorescens (B77) strains and combined nitrogen at field applied dose on nodulation of Vicia faba inoculated with four strains of Rhizobium leguminosarum bv. viceae isolated from Hungary (Bükköny 75/4 and Lóbab Z), England (1012) and Libya (3841-HB). Inoculation of sterilized brown forest soil (Gödöllő) with Rhizobium strains relatively increased the nitrogen content, dry matter and height of the plants. Application of inorganic nitrogen as NH_4NO_3 increased both total nitrogen content and dry matter of the plants rather than the inoculation with P. fluorescens. The number of root nodules in all Rhizobium treatment was more in Rhizobium-Pseudomonas combination than in Rhizobium-NH₄NO₃ treatments. The plant height was not changed in both combinations. The growth of plants were at maximum in Rhizobium-Pseudomonas-NH₄NO₃ combination. Soil inoculation with P. fluorescens increases the level of nodulation of invaded plants with Rhizobium. The tested strains were not inhibited by P. fluorescens.

P. SIPOS and M. KECSKÉS

Root nodulation of soybean by Bradyrhizobium japonicum on the effect
of N-fertilizer doses

Department of Microbiology, Agricultural University, Gödöllő, Hungary

The compatibility of inoculation of soybean (Glycine max cultivar Imo-1a) by five Bradyrhizobium japonicum strains and different N-fertilizer doses (30, 60, 120 kg N/ha) was studied in small plot (55 m²) field experiments (acid brown forest soil, pH 5.3). Nodules formed only on the roots of inoculated plants. Number of nodules was the biggest on roots of plants of control plots, but the decrease in number was not significant in the 30 and 60 kg N/ha treatments. One hundred and twenty kg N/ha N-doses inhibited the nodule formation on decreased significantly to compare to the control. The highest yield (seeds) was produced in the 120 kg N/ha treatments, but the yield was higher in the 30 kg N/ha treatments in the case of three strains than in plots with 120 kg N/ha. As "starter" nitrogen, 30-40 kg N/ha can be used, which does not alter the soybean-bradyrhizobium symbiosis.

H. DOMJÁN KOVÁCS, V. PINTÉR TABAJDI and B. RALOVICH

Paralell examination of food samples for Listeria selective isolation
and ELISA test

Laboratory of Veterinary and Food Investigation Institute, Budapest, and
Hungarian Meat Research Institute, Budapest, Hungary

A total of 272 ripened and dried raw meat products were examined for Listeria. Two kinds of enrichment as well as selective procedures and also two ELISA kits were tested. In the first procedure UVM₁-UVM₂-LPM agar and in the other method UVM₁-Fraser broth-modified Oxford medium were applied. Out of 272, 183 samples were positive by both enrichment and cultivation methods. Besides, further 10 samples were positive by the first procedure (total 71%), and further 29 samples by the other method (total 77.9%). Of the 272 products 31 samples were tested parallel with the NOACK ELISA kit and other 40 samples with the ORGANON ELISA kit. The second cultivation

method was more effective than the first procedure. For the Fraser broth 48 h incubation was necessary. Both ELISA kits required 10^4 listeriae in the enrichment medium for a positive reaction.

H. E. A. F. BAYOUMI and M. KECSKÉS

Growth of Rhizobium leguminosarum bv. viceae strains and their symbiosis with Vicia faba affected by some soil applied pesticides

Department of Microbiology, Agricultural University, Gödöllő, Hungary

The effect of Paraquat, Irifluralin, Dithane M-22, and Orthocide at field recommended rate on the growth of effective Rhizobium leguminosarum bv. viceae (Bükköny 75/4, Lóbab Z, 3841-HB, and 1012) strains and their symbioses with Vicia faba (Libyan variety) was studied using microfermentor and pot experiment techniques. In the course of our comparative investigation carried out in the pure and ecosystem cultures, it was established that of the symbionts the microsymbionts are more sensitive to Dithane M-22 and Tri-fluralin than the macrosymbiont, whereas both of them are sensitive to Paraquat and Orthocide. Application of Paraquat and Orthocide was found to reduce the size, number and dry weight of root nodules, phytobiomass, height and total nitrogen content per plant significantly. The invaded plants with Bükköny 75/4 and Lóbab Z rhizobial strains are more tolerant to these soil applied pesticides comparatively than those invaded by other strains.

B. TH. Le and CS. DOBOLYI

Survival of two selected Trichoderma strains in pepper and cucumber rhizosphere

Department of Microbiology, Agricultural University, Gödöllő, Hungary

The survival of highly antagonistic Trichoderma viride and Trichoderma harzianum strains was investigated in the rhizosphere of pepper (Capsicum annum L.) and cucumber (Cucumis sativus L.) plants. In pot experiments in sterile brown forest soil the survival of both Trichoderma species was long and characteristic. The seed treatment moderately influenced the capability

of germination: e.g. on the effect of T. viride the germination capability was of 95.0%, on the effect of T. harzianum it was of 92.5%, while it was of 83.8% in untreated seeds. The population of the used Trichoderma strains was higher in the rhizosphere after seed treatment than in non-rhizosphere soil. On the other hand, the population of them was lower in the rhizosphere after soil-treatment than in non-rhizosphere soil. On the basis of survival of both Trichoderma strains in the rhizosphere a true rhizosphere compatibility may be assumed.

I. C. SHUKLA and A. K. PANDEY

Studies on phorate resistant mutants

Department of Chemistry, University of Allahabad, Allahabad, India

The present paper describes studies on phorate resistant mutant strains of chick pea (Cicer arietinum) Rhizobium sp. growth and their compatibility in symbiotic nitrogen fixation. It shows the effect of higher dose of phorate on growth and compatibility of symbiotic nitrogen fixation of parent strain RG3, phorate resistant mutant strains G11, G26 as well as ineffective indigenous rhizobium strains. A pure culture of Rhizobium strain RG3 was isolated from a root nodule of chick pea variety C235 and grown in yeast-mannitol broth (0.5 g K_2HPO_4 ; 0.2 g $MgSO_4$; 0.1 g NaCl; 2 g $CaCO_3$; 10 g mannitol; 10 g yeast extract in 1 litre distilled water). The pH was adjusted to 7.5 and the broth solidified with 1.5% (w/v) agar when required. A fresh culture of this strain in yeast extract mannitol (YM) broth was washed and suspended in phosphate buffered saline (PBS, pH 7.2). Two ml of the culture containing 6.4×10^6 colony forming units (c.f.u./ml) was treated with 30 μ g N-methyl-N-nitro-N-nitrosoguanidine (MNNG/ml) for 20 min at 35 °C. Another 2 ml culture was kept as an untreated control. Both cultures were washed and diluted separately in 15 ml YM broth and shaken overnight at 28 °C. The two cultures were then washed and resuspended in 2 ml YM broth of which 0.2 ml ($1.8-2.6 \times 10^8$ c.f.u.) was plated on yeast mannitol agar containing 200 μ g phorate/ml and incubated at 28 °C. Within 7 days of incubation six colonies were found on the 2000 μ g phorate/ml plates. These colonies were grown separately in YM broth culture tubes, purified and grown twice in 2000 μ g phorate/ml. These mutants were designated as G-7, G-9, G-11, G-13, G-22 and G-26. The frequencies of mutation was 6×10^{-7} .

For growth studies the strains were grown at 28 °C for 1 week in shaken culture containing technical grade phorate (diethyl phosphorodithionate) to give final concentration of 500, 1000, 1500 and 2000 µg/ml. These mutant strains were also tested for nodulation on agar slants with chick pea variety C235 as the test plant. Only G11 and G26 retained the ability to form nodules and were used in the field trial. On the basis of number of nodules, their dry weight, nitrogenase activity, total pyridine nucleotides and active iron contents, mutant strains G11 and G26 were found to be equally effective with and without phorate and resulted in significantly greater grain yield.

M. PESTI

Inheritance of Verticillium dahliae in Capsicum ssp.

Research Center of VETÜMAG Seed Producing and Trading Enterprise, Szentes, Hungary

The testing of 24 Verticillium isolates originating from different regions of Europe revealed that Verticillium dahliae, Verticillium alboatrum and Verticillium tenerum might be pathogenic to Capsicum annuum varieties. Verticillium isolates from artichoke, eggplant and tomato also proved to be pathogenic to pepper. Only 15 of 115 lines and cultivars of pepper behaved as a heterogeneous population, indicating a resistance level in the range 3–20%. Inbred lines of Podarok Moldavii variety were selected through 3 generations by applying the most aggressive V. dahliae isolates. This procedure did not result in an increased resistance of the selected lines, indicating the polygenic nature of the inheritance of V. dahliae resistance in this cultivar.

E. GEDE and S. N. KÓSA

Antimicrobial agents produced by Pseudomonas strains

Technical University, Budapest, Hungary

The antagonism between several Pseudomonas strains and phytopathogenic bacteria/fungi was examined (in vitro). Pseudomonas aurantiaca BKM 875 and Pseudomonas fluorescens NCIB 10586 were active against a variety of phytopa-

thogenic fungi. These strains inhibited the growth of Penicillium glaucum, Fusarium moniliforme and Stemphylium radicinum. P. fluorescens produced antibacterial substance, too, which inhibited the growth of Xanthomonas campestris and Bacillus subtilis. A new fast method was elaborated for testing the antimicrobial activity. Extracts prepared from the yeast extract or King B medium in which Pseudomonas strains were grown was applied to Kiesel-gel 60 F₂₅₄ thin-layer chromatography plates and run in the solvent chloroform-methanol (1:1, v/v). Two fluorescent spots appeared with R_f values of 0.67 and 0.82. After chromatography the plates were sprayed with PDA broth containing spores of P. glaucum. After 2-3 days at 27 °C in a sterile chamber, the zones of growth inhibition of the fungus were recorded with the same R_f values which all goes to show that these spots correspond to the antibacterial substances.

S. BALÁZSY and M. KECSKÉS

Some ecophysiological characteristics of
Bradyrhizobium sp. (Lupinus) strains

Department of Botany, Gy. Bessenyei Teacher's College, Nyíregyháza, and
Department of Microbiology, Agricultural University, Gödöllő, Hungary

Three white lupin root nodule strains were selected for investigation from strains isolated from Lupinus albus grown in Nyírség (Hungary) in acid brown forest soil (pH 5.0). Some ecophysiological characteristics of these were compared to other Rhizobium strains. Microsymbionts originating from root are normally slow-growing; our three effective strains proved to be fast-growing possessing multiflagella and they produced acid in synthetic medium. The generation time of these were from 3.4 to 4.0 h at pH 6.0 and 7.0 and 3.5 and 4.2 h at pH 8.0. The strains had different optimum pH for growth. They could survive the cold (-24 °C) and hot (40 °C-55 °C) stresses. Our strains are similar to the other fast-growing Rhizobium strains.

J. KUKOLYA and F. FELFÖLDI

Localization and partial characterization of beta-D-glucosidase
(true cellobiase) produced by a species of Cellulomonas

Department of Microbiology, Department of Agricultural Chemistry and Biochemistry,
Agricultural University, Gödöllő, Hungary

A species of the coryneform bacteria Cellulomonas produces strictly cell-bound beta-D-glucosidases: aryl-glucosidase, and true cellobiase. True cellobiase is one of the components required for the efficient enzymatic conversion of cellulose to glucose. The surface-bound enzyme hydrolysed p-nitrophenyl-beta-D-glucoside (PNPG) and cellobiose. The molecular weight of beta-D-glucosidase was 45 000. Temperature optimum was 44 °C, and the enzyme showed optimal activity at pH 6.9. The results of kinetics studies: K_m was 1.06×10^{-3} M for PNPG, V_{max} 6.29 nMPNP/min. Beta-glucosidase activity when PNPG was used as substrate was inhibited competitively by delta-gluconolactone with a K_i value of 1.05×10^{-5} M. The K_i for glucose, a competitive inhibitor, was 1.8×10^{-3} M.

M. J. ROMÁN

Occurrence of lactic acid bacteria in Hungarian wines

Department of Microbiology and Biotechnology, University of Horticulture and Food Industry,
Budapest, Hungary

Lactic acid bacteria are important in wine making for two major reasons. Some of them are involved in so-called wine diseases, for example the fermentation of fructose to mannitol, or the fermentation of glycerol to acrolein. However, some species of lactic acid bacteria are able to convert malic acid to lactic acid. This malo-lactic fermentation not only reduces the acid content but also has an influence on the flavour. Therefore depending on the technique of processing, the malo-lactic fermentation is regarded as either favourable or a nuisance and is inhibited because of unwanted effects on the wine. Having identified 120 strains of lactic acid bacteria from Hungarian wines, we determined the most frequently occurring species. Leuconostoc oenos was most active in red wines.

A. POMÁZ, A. TÓTH, Á. SZÉCSI and L. HORNOK

Toxin producing Fusarium species from Hungarian fodder and grain samples

Agricultural Biotechnology Center, Gödöllő, Department of Plant Protection, Agricultural University, Gödöllő, and Plant Protection Institute, Hungarian Academy of Science, Budapest, Hungary

Fodder and wheat grain samples from different regions of the country were plated on fusarium-selective medium. More than 15 species were identified from this collection. Samples that had not been sterilized superficially, yielded mainly two fungi: Fusarium moniliforme and Fusarium moniliforme var. subglutinans. When surface sterilized grains were plated, several species of the Arthrosporiella-Sporotrichiella sections were isolated; these species are regarded as the most dangerous trichothecene producers. According to our survey Fusarium poae seems to predominate among them. Thin-layer chromatography on fluorescent silica gel plates was applied for toxin identification using Sigma preparations as standards. The majority of the isolates produced one or more representatives of the five most important Fusarium mycotoxins (zearalenone, diacetoxyscirpenol, nivalenol, deoxynivalenol, T-2 toxin). The fungal isolates formed distinct chemotaxonomical groups on the basis of their toxin production patterns.

E. SZÁLLÁS, ZS. KOMLÓ, L. NAGY, J. KOZMA, A. FODOR and A. SZENTIRMAI

Specific association between bacterium and insectpathogenic nematodes

Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, and Department of Genetics, L. Eötvös University, Budapest, Hungary

A few genera of rhabditoid nematodes could be used for biological control of range of insect pests. Nematodes are usually dwelling in soil, they can survive several months, even years without feeding. It is easy to select entomopathogene nematodes, using Galleria mellonella larvae as the bait insect within two weeks multiply in the grubs (a few hundredthousands infective larvae per gram cadaver). During last decade, a number of bacterial symbionts were isolated from insectpathogenic nematodes. The infectivity of nematodes is markedly influenced by the associated bacteria. However, the symbiont bacteria increase the rate propagation of nematodes. To produce

nematodes in aerated stainless steel vessel is an economic way. It is inoculated with shaken flask culture of the symbiont bacterium. After two day growing period 200 nematodes per ml fermentation source were transferred into the fermentor. More than 50 thousand infective nematodes per ml fermentation broth can be obtained after 3-4 weeks growing period.

CS. DOBOLYI and T. CSELÉNYI

Incidence of different microbe populations on surfaces of legume
and cereal plants

Department of Microbiology, Agricultural University, Gödöllő, Hungary

The population dynamics, the species diversity and some ecophysiological characteristics of the epiphytic microbes living on the surface of leguminous and cereal plants were studied. The existence of different fungal and bacterial populations was significant: 10^5 – 10^6 yeast, 10^4 – 10^5 mould and 10^6 Pseudomonas propagules could be cultivated as an optimum of the vegetation period. All of them showed regular seasonality and some other ecological features. The persistence of Azotobacter cells disposed on phyllosphere exceeded three weeks and depended on the preventing agent applied. The most frequent yeast genera were Aureobasidium, Cryptococcus, Sporobolomyces and Rhodotorula in the phyllosphere, while Cryptococcus, Candida and Rhodotorula in the rhizosphere. The members of Lipomycetaceae were present in the rhizosphere. None of isolated yeasts could ferment sugars, whereas they had some catabolic activities and antagonistic properties against each other.

B. HELMECZI and J. KÁTAI

Effect of organic, artificial and lime fertilization
on microbiological activity in soil

Department of Soil Science and Microbiology, Agricultural University, Debrecen, Hungary

Comparative soil microbiological tests were carried out in a small plot plant-production in which combination of different amounts of manure, fertilizer and lime were applied. The number of total bacteria and microscop-

pic fungi was determined by plate dilution method, the quantity of cellulolytic and nitrifying bacteria by Pochon-Tardieux's method (1972). Cellulolytic activity was measured with Unger's (1968), and CO_2 -production was determined by Witkamp's (1966 in Szegi, 1979). Enzymes activities in the soil (phosphatase, saccharase, urease, catalase) were investigated on the basis of Szegi's collected methods (1979). Fertilization stimulated the quantity of cellulolytic bacteria and phosphatase activity first of all but slightly increased the amount of total and nitrifying bacteria, too, as well as the cellulolytic activity, CO_2 -production and urease activity. Liming slightly increased the quantity of the nitrifying bacteria and CO_2 -production of all the microbiological characteristics examined but decreased phosphatase and saccharase activities. Microbiological activity was stimulated with combined applications of organic and lime fertilization except for microscopic fungi, cellulolytic bacteria and phosphatase activity.

A. BINNYEI, S. TIMÁRI and M. KECSKÉS

Cellulose pearl as a carrier for Bradyrhizobium inocula

Interprotein and Biotechnology Ltd, Budapest, and
Department of Microbiology, Agricultural University, Gödöllő, Hungary

A new type carrier was investigated for soybean's seed inoculation of Bradyrhizobium japonicum strains. Pot experiment was carried out with soybean (Glycine max L. cultivar Imola) in glasshouse having light, heat and moisture regulation. Acidic (pH 5.4) poor in humus (1.2%) rostr brown forest soil was used. Inocula were prepared from Bradyrhizobium japonicum effective strains using "MAVI" cellulose pearl. These cellulose pearls containing bradyrhizobium cells were placed besides the soybean seeds in different amounts. Number and weight of root nodules, the number and mass of pods, as well as the length of roots and upper part of plants were considered.

We found that (i) the inocula with cellulose pearls increased the number of nodules significantly, but in the mass of nodules were not differences; (ii) the mass of roots was not changed markedly on the effect of inoculation; (iii) some treatments (3 cellulose pearls/seed) increased the mass of the upper part of soybean significantly, others resulted higher mass and number of pods.

Z. MIJAČEVIĆ, L. STOJANOVIĆ and D. IVANOVIĆ

The influence of disturbed secretion on milk structure,
cheese quality and on growth of microorganisms

Veterinary Faculty, Beograd, and Agroekonomik Co., Padinska Skela, Yugoslavia

Total milk with number of cells of 2×10^5 and 7×10^5 /ml was chosen for cheese production. By chemical analyses it was found out that the percentage of dry substance, fats and casein was smaller and the percentage of serum proteins greater in the milk with greater number of cells. The cheese quality depended directly on the number of cells in milk. The cheese obtained from milk with 7×10^5 cells/ml had greater humidity and smaller dry substance than the control cheese. During initial stages of cheese production it was noticed that in milk with greater number of cells growth of microorganisms was more intensive in the first part of production.

J. KÁTAI and B. HELMECZI

Comparative evaluation of enzyme activity in some Hungarian soil types

Department of Soil Science and Microbiology, Agricultural University, Debrecen, Hungary

Under natural conditions humification and mineralization in the soil are determined by the important physical and chemical characteristics of the soil as well as climatic factors. Depending on these characteristics the amount and intensity of microorganisms and their enzyme activity varies in the different soil types. The activity of four enzymes (phosphatase, saccharase, urease, catalase) of the sixteen more frequent soil types of the Debrecen region was compared. Enzyme activity in the soils was measured on the basis of Szegi's collected methods (1979). Of the soils with extreme reaction, lower activity values were observed on acidic humic sand, brown forest soil with clay layers and alkaline solonchak-solonetz with all the four enzymes. Phosphatase and catalase activity was significantly greater in brown forest soil than that described by Ramann. With clay illuviation, in chernozem and humic alluvial soils average activity values were observed. Phosphatase and saccharase activity values proved to be high while those of

urease and catalase low in meadow solonetz turning into steppe formation. In solonchak- and solonetz-like meadow soils high urease activity and very low phosphatase and saccharase activity values were measured.

With all the four enzymes very high activity was observed in marshy meadow soil with light structure and high organic matter content. Activity values proved to be high with phosphatase and low with the other three enzymes in heavier marshy meadow and seadow soils.

Z. NAÁR and M. KECSKÉS

Strains of Trichoderma spp. antagonistic against some broad bean's pathogens

Department of Microbiology, Agricultural University, Gödöllő, Hungary

Thirty five Trichoderma strains of T. hamatum, T. harzianum, T. konin-gii, T. longibrachiatum, T. viride species were studied against 9 broad bean's pathogen strains of Alternaria sp., Cladosporium herbarum, Fusarium spp., Fusarium oxysporum, Fusarium semitectum, Penicillium spp., Tricho-thecium roseum on potato-dextrose agar. Eleven strains were selected on the basis of dual culture and they were tested with cellophane-method for anti-fungal and mycoparasitic potential, respectively. The Trichoderma strains were generally effective against pathogens; T. hamatum, T. longibrachiatum, T. harzianum, and T. viride strains were the most effective.

T. roseum was the most resistant pathogen, and Alternaria sp. proved to be the most sensitive.

LACTOFERRIN SYMPOSIUM

A. S. NAIDU

Lactoferrin and the preimmune host defence

Department of Medical Microbiology, Malmö General Hospital, University of Lund, Malmö, Sweden

Breast-feeding protects the infants against gastroenteritis. Various antimicrobial components in milk, lactoferrin (Lf) in particular, have been attributed to this prophylaxis. Lf is an iron-binding protein secreted by polymorphonuclear leukocytes as well as various exocrine glands and is abundant at the mucosal surface. This molecule plays an important role in host defence during inflammation or endotoxin shock, and suggested to regulate iron absorption in the gut. Lf elicits bacteriostatic, bactericidal, and opsonic effects on bacteria. Specific Lf receptors are present in the antigen-processing cell cascade i.e. monocytes, macrophages, polymorphonuclear leukocytes, B-lymphocytes and activated T-lymphocytes. Lf also participates in the regulation of GM-CSF and antibody synthesis. From our laboratory, we have recently described specific receptors for Lf on various bacterial pathogens. Due to the recent developments and the increasing accumulation of knowledge on structure/function, it is necessary to reevaluate the loose ends in this area of research and to propose a new definition for lactoferrin molecule.

J. ERDEI, A. FORSGREN and A. S. NAIDU

Lactoferrin binding outer membrane proteins in Escherichia coliInstitute of Pathophysiology, University Medical School, Debrecen, Hungary, and
Department of Medical Microbiology, Malmö General Hospital, University of Lund, Malmö, Sweden

Certain strains of Escherichia coli avidly bound Lf, while a majority of clinical isolates demonstrated low or no interaction. Unexpectedly, the western-blots revealed Lf-binding outermembrane proteins (Omp) in both Lf-binding and non-binding strains. The bacterial receptors for Lf appeared between 30 and 40 kD region, in heat treated (100 °C) Omp preparations and

these bands disappeared in unheated Omp preparations. Porins OmpC and OmpF were suspected as Lf binding receptors in E. coli. Constructs for porin expression, demonstrated characteristic patterns for OmpC, OmpF and PhoE. OmpF was purified to homogeneity, and this porin in both trimeric (90-120 kD) and monomeric forms, reacted with specific mAbs and Lf, in western-blot assay. These data establish a specific binding of Lf to porins in E. coli, and the LPS in smooth form seem to shield the porins from Lf interaction with bacteria.

S. S. NAIDU, J. ERDEI, E. CZIRÓK, I. GADÓ, I. TÓTH,
A. FORSGREN and A. S. NAIDU

Lactoferrin binds to Escherichia coli and other Gram-negative bacteria
associated with human intestinal infections

Department of Medical Microbiology, Malmö General Hospital, University of Lund, Malmö, Sweden,
Institute of Pathophysiology, University Medical School, Debrecen, and
B. Johan National Institute of Hygiene, Budapest, Hungary

Escherichia coli associated with human intestinal infections bound HLf and BLf in the range of 3.7 to 73.4% and 4.8 to 61.6%, respectively. ETEC strains demonstrated a significantly higher HLf binding than EPEC, EIEC and EHEC strains. EPEC strains belonging to serotypes O44 and O127 demonstrated significantly higher HLf binding compared to O26, O55, O111, O119 and O126. The Lf binding mechanism was further characterized with an EPEC strain, E34663 (serotype O127). The binding was stable in the pH range 4.0 to 7.5 and did not dissociate in the presence of 2 M NaCl or 2 M urea. The binding reached a complete saturation within 110 min. The interaction could be displaced in a dose-dependent manner, by unlabelled HLf or BLf. Apo- and iron saturated-forms of Lf showed similar binding. Binding-inhibition with various proteins and carbohydrates suggested a specific nature of the interaction. Scatchard-plot analysis gave 5400 specific HLf receptors/cell, with an affinity constant (K_a) 1.4×10^{-7} M.

Z. TIGYI, A. FORSGREN and A. S. NAIDU

Lactoferrin-binding proteins in Shigella flexneri

Institute of Medical Microbiology, University Medical School, Pécs, Hungary, and
Department of Medical Microbiology, Malmö General Hospital, University of Lund, Malmö, Sweden

Shigella flexneri strain M90T was characterized for lactoferrin binding. The binding of ^{125}I -HLf and ^{125}I -BLf to strain M90T reached a saturation within 2 and 5 h, respectively. Unlabelled HLf and BLf were recognized by bacteria, and elicited a displacement of ^{125}I -HLf-bacteria interaction in a dose-dependent manner. Transferrin, the major iron-binding protein in plasma inhibited Lf binding. The binding inhibition with hemin, sodium deoxycholate, and carbohydrates: GluNac, GalNac, NANA and NAMA was $<40\%$. Congo red caused 52% and 35% inhibition of labelled HLf and BLf binding. The interaction was specific with dissociation constant (K_d) values of -1.2×10^{-7} L/mole and -9.3×10^{-8} L/mole for HLf and BLf, respectively. According to Scatchard analysis, approximately 4800 (for HLf) and 5700 (for BLf) binding sites per cell were estimated on strain M90T. The presence of Lf-binding components in bacterial cell envelope (CE) and outermembrane (OM) of strain M90T (crb+) and M90T55 (crb-) was examined in western-blot studies. Unheated CE and OM preparations were negative for Lf binding. However, after heating, three distinct bands of approximately 39-kD, 22-kD and 16-kD were identified as Lf-binding components, and the patterns were similar for both BLf and HLf, in crb+ and crb- strain pair.

F. ROZGONYI, Z. TIGYI, A. FORSGREN and A. S. NAIDU

Lactoferrin potentiates cefuroxime effect in Salmonella species

Institute of Microbiology, University Medical School, Debrecen, Hungary, and
Department of Medical Microbiology, Malmö General Hospital, University of Lund, Malmö, Sweden

The interaction of human and bovine lactoferrins (HLf and BLf) with 220 Salmonella (39 different species comprising 10 serogroups) isolated from gastroenteritis patients and healthy food handlers, was tested in an isotope-labelled protein binding assay. Most isolates showed a moderate (15 to 30%) to a high ($> 30\%$) degree of Lf binding. Salmonella hartford strain

HNCMB 10063, bound 23.1% and 27.8% of ^{125}I -HLf and ^{125}I -BLf, respectively, and showed a time-dependent binding saturation within 90 min. Apo-BLf at 1 mg/ml concentration did not change the growth of strain HNCMB 10063. Cefuroxime at 4 $\mu\text{g/ml}$ and 8 $\mu\text{g/ml}$ concentration altered the growth kinetics of the above strain by lengthening the lag-phase for 9 and 21 h (followed by a short log-phase), respectively, and ultimately inhibited the cell multiplication. However, both concentrations of cefuroxime, in combination with apo-BLf (1 mg/ml) elicited a synergistic effect and completely inhibited the bacterial growth. Thus, interaction of Lf with bacteria seem to potentiate in vitro antimicrobial action of cefuroxime.

É. CZIRÓK, M. HERPAY, A. PINTÉR, I. GADÓ, H. MILCH and A. S. NAIDU

Effect of lactoferrin on the intertinal colonization of
Escherichia coli in a mouse model

B. Johan National Institute of Hygiene, Budapest, Hungary, and
Department of Medical Microbiology, Malmö General Hospital, University of Lund, Malmö, Sweden

The effect of lactoferrin (Lf) on the intestinal colonization of Escherichia coli strain F18A⁻ and strain F18 Col⁻ was investigated in a mouse model. The group of animals that received Lf in combination with streptomycin (Str), have demonstrated a significantly low degree of bacterial bowel colonization, compared to Lf treated or untreated groups. Str treatment alone had no influence on the colonization. Furthermore, Lf has completely blocked the binding of subepithelial matrix proteins to E. coli strain E1 in vitro. In competitive binding-displacement studies. Lf (10 mg/ml) has effectively dissociated the bacterial interaction with collagen type-I and laminin, but not the interaction with fibronectin, fibrinogen and collagen type-IV. Pathomorphological changes during bacterial colonization in vivo and molecular aspects of Lf interference with subepithelial matrix protein binding to bacterial in vitro shall be discussed.

1. GADÓ, V. G. LÁSZLÓ, J. ERDEI, J. PÁSZTI, É. CZIRÓK,
H. MILCH and A. S. NAIDU

Lactoferrin binding and colicin sensitivity in Escherichia coli

B. Johan National Institute of Hygiene, Budapest, Hungary, and
Department of Medical Microbiology, Malmö General Hospital, University of Lund, Malmö, Sweden

Escherichia coli strain H10407 demonstrated a low Hlf binding (7%) and was insensitive to group A as well as group B colicins. A spontaneous Hlf high binding (44%) variant H10407 (Lf) demonstrated an increased sensitivity to both colicin groups. On the other hand, colicin-insensitive E. coli wild-type strains 75ColT, 84ColT and 981ColT showed a low degree of Hlf binding i.e. 4%, 8% and 10%, respectively. The Hlf binding capacity was high in the corresponding colicin-sensitive mutants 75ColS (43%), 84ColS (32%) and 981ColS (43%). The Hlf binding capacity and susceptibility to colicins has enhanced, when bacteria were grown in media containing EDTA. These data establish a strong correlation between Hlf binding and colicin susceptibility and the involvement of LPS seems to be a common feature for both phenomena.

A. R. KISHORE, J. ERDEI, A. FORSGREN and A. S. NAIDU

Enzyme-linked ligand binding assay (ELBA) for the detection of
lactoferrin binding in bacteria

Department of Medical Microbiology, Malmö General Hospital, University of Lund, Malmö, Sweden,
and B. Johan National Institute of Hygiene, Budapest, Hungary

^{125}I - or ^{59}Fe -labelled lactoferrins (Lf) are widely used in binding studies with bacteria or mammalian cells. The labelling, handling and disposal of isotope-labelled proteins requires special expertise, containment and equipment. Moreover, labelled proteins are unstable due to radioactive disintegration. Therefore, an enzyme-linked immunosorbent assay (ELBA) was developed at our laboratory to examine the Lf interaction with bacteria. Lf was coupled to horseradish peroxidase (HRPO) and the enzyme-labelled ligand and has been titrated for optimal reaction, using E. coli strains H10407 (negative control) and mutant H10407(Lf) (positive control). The standard-

ized ELBA was evaluated on > 400 strains of E. coli belonging to various clinical sources. The ELBA extinction values were compared with ^{125}I -labeled Lf binding assay values for individual strains. Regression analysis gave a good correlation between the two assays. ELBA was also suitable for performing Lf binding saturation kinetics, binding-displacement experiments. This newly developed ELBA seems to be an alternative semi-quantitative detection system for Lf interaction with bacteria.

A. S. NAIDU, J. L. WATTS and A. FORSGREN

Bovine lactoferrin receptors in staphylococci causing mastitis

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and Animal Health Therapeutics Research, The Upjohn Co., Kalamazoo, USA

Bovine lactoferrin (BLf), bound to Staphylococcus aureus and to six common mastitis-associated species of coagulase-negative staphylococci. The BLf receptor density per bacterium and the binding affinity constants (in L/mole) for different species was as follows: S. aureus (7200, $K_a = 14 \times 10^6$); Staphylococcus epidermidis (3600, $K_a = 11.9 \times 10^6$); Staphylococcus warneri (1900, $K_a = 3.1 \times 10^6$); Staphylococcus hominis (4100, $K_a = 3.8 \times 10^6$); Staphylococcus xylosus (4400, $K_a = 3.3 \times 10^6$); Staphylococcus hyicus (6100, $K_a = 1.0 \times 10^6$); and Staphylococcus chromogenes (4700, $K_a = 2.5 \times 10^6$). Two proteins with an estimated molecular weight of approximately 92-kDa and 67-kDa were identified as BLf binding components in S. aureus strains SA-340.

S. KALFAS, M. ANDERSSON, S. EDWARDSSON, A. FORSGREN and
A. S. NAIDU

Interaction of lactoferrin with periodontitis-associated bacteria

Department of Oral Microbiology, School of Dentistry, University of Lund, Malmö, and
Department of Medical Microbiology, Malmö General Hospital, University of Lund, Malmö, Sweden

Human lactoferrin (HLf) concentration in parotid saliva is $\sim 10 \mu\text{g/ml}$, and its level significantly rises during localized juvenile periodontitis, gingivitis and adult periodontitis. We have investigated for the specific

interaction of Lf with human isolates of periodontitis-associated black pigmented Porphyromonas gingivalis, Prevotella intermedia and Prevotella melaninogenica, P. intermedia exerted a high Lf binding and the interaction was specific with a high affinity (K_a , 5.5×10^{-7} M). Lf binding seemed to be non-specific in P. gingivalis and P. melaninogenica. The binding components in all species were thermolabile and protease sensitive. The results suggest that Lf binding in these species is distinct from the previously described interactions with plasma and subepithelial matrix proteins.

ELEVENTH CONGRESS OF THE HUNGARIAN SOCIETY
FOR MICROBIOLOGY

PAPERS PRESENTED AT THE

FLUOROCULT SYMPOSIUM

ORGANIZED BY THE

HUNGARIAN SOCIETY FOR MICROBIOLOGY

and E. MERCK

BUDAPEST, HUNGARY, AUGUST 24, 1991

INTRODUCTORY REMARKS TO THE FLUOROCULT SYMPOSIUM

G. HOFMANN

Mr. Chairman, Ladies and Gentlemen:

On behalf of the E. Merck Darmstadt Germany it is my pleasure to welcome you to our Fluorocult Symposium. Fluorocult was already the main topic of a Symposium in 1988, when microbiologists met in Würzburg, Germany, presenting their first practical experience to detect Escherichia coli by use of Fluorocult media containing 4-methylumbelliferyl-beta-D-glucuronide (MUG) as substrate. The speakers in Würzburg have shown the reliability of the fluorogenic E. coli assay in medicine as well as in the examination of water and in the food processing industry. In addition they pointed out that the use of solid and liquid media containing MUG facilitates the diagnostic procedure which is therefore less laborious and time consuming than conventional methods and which enables conclusive results to be achieved much faster. The superiority of the fluorogenic procedure over conventional methods was said to be most apparent when E. coli need enriching. For example, tests for E. coli are variously required within the dairy industry. In Germany, these are mostly carried out with Brilliant Green Bile Lactose Broth (BRILA Broth), though in other countries Lauryl Sulphate Broth is more commonly used. In addition to the classical criteria of "growth" and "gas formation through lactose fermentation", Fluorocult broths enable the factors "MUG cleavage by beta-D-glucuronidase" and "production of indole from tryptophan" to be evaluated. Organisms are reliably identified after just a single enrichment.

Since 1988 there must have been generated more experiences in more technical and regional areas of the fluorogenic E. coli detection. The widening of the regional areas is symbolized in two papers presented at the

GOTTFRIED HOFMANN
E. Merck Diagnostic Division
Research and Development
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IUMS-Congress in Osaka last year and in our symposium of today being here in Budapest with the participation of lecturers coming from Austria, Japan, Germany and Hungary. The widening of technical experiences will be shown again in medicine as well as in the examination of water and in the meat and milk processing industry. In addition we will get information of the development of a fluorogenic medium designated for E. coli 0157:H7 and an overview to the different fluorogenic substrates and their potential for detection of different microorganisms.

I should like to thank Dr. Béla Ralovich for taking over the chairmanship. I say thank you to all who make this symposium possible and who are now going to share with all participants their expert knowledges in the fluorogenic detection of E. coli.

DETERMINATION OF TOTALCOLIFORM AND FAECALCOLIFORM
BACTERIA FROM BATHING WATER WITH FLUOROCULT-BRILA-BROTH*

G. HAVEMEISTER

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(Received September 24, 1991)

The BRILA-MUG one-tube-test in connection with the MPN-method can be used successfully to determine with sufficient reliability the number or indices of total coliforms and faecal coliforms according to the EC-Directive for bathing waters. Using the one-tube-test and determination of gas production, fluorescence and indole production is, from the hygienic point of view for surface waters, equal in value to subculture and biochemical identification. The test needs only a minimum of material and laboratory staff. Differences between this test and other more extensive tests with several biochemical identification steps are negligible. The work load for bathing water monitoring would not be justified in this case. Due to the occurrence of Aeromonas, the values for total coliforms compared to a determination with biochemical identification are higher in some cases. A high contamination with Aeromonas indicates a high polluted surface water with a high degree of eutrophication. This is an important additional criterion for the evaluation of bathing waters, even when a direct dependence on faecal contamination does not exist in every case. The degree of an up-to-date faecal contamination can be estimated by the pollution with faecal coliforms.

In the highly civilized areas of Europe and especially in places with a high density of tourism, no surface waters exist without sewage pollution. Since there are always persons in the population who excrete pathogenic bacteria, every surface water has to be considered as infectious. In connection with regular monitoring of bathing waters it is not possible to carry out an analysis of all bacteria which theoretically are able to cause an infectious disease, i.e. pathogenic germs from the intestinal tract like Salmonella, Shigella, Vibrio cholerae, etc. and also some kinds of enteroviruses. Usually the examination of water therefore includes only the detection of so-called bacterial indicators for pollution. A possible risk of infection is directly proportional to a demonstrable faecal contamination /1/.

G. HAVEMEISTER

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*Dedicated to Mr. H. Gärtner, Dr. and emer. Professor for Hygiene and Microbiology to his 80th birthday.

In the areas of the European Community (E.C.) the E.C. Directive 76/160/EEC (concerning the quality of bathing water) is valid for the sanitary assessment of bathing waters /2/. This guideline prescribes to examine samples from surface waters at constant intervals of time (normally fortnightly) and to determine the parameters total coliforms and faecal coliforms (testing of other microbiologic parameters is necessary only in special situations). As shown in Table I, for the evaluation there are guide values (G) and mandatory or imperative values (I).

Table I
Microbiological guide values (G) and mandatory or imperative values (I),
According to the E.C. Directive for Bathing Water

Parameter		Guide value (G)	Imperative value (I)
Total coliforms	/100 ml	500 (80)	10000 (95)
Faecal coliforms	/100 ml	100 (80)	2000 (95)
Faecal streptococci	/100 ml	100 (90)	—
Salmonellae	/ 1 l	—	0 (95)
Enteroviruses	/ 10 l	—	0 (95)

Numbers in round brackets do mean the number of samples with values which have to be not higher than G- respectively I-values!

Materials and methods

The E.C. Directive contains methods of analysis. For the analysis of total coliforms and faecal coliforms the E.C. Directive allows two alternative methods (Table II).

According to the MPN (Most Probable Number) method with fermentation in multiple tubes it is necessary to apply at least 9-15 dilution tubes for one sample to cover the range between guide and mandatory values. From highly contaminated water samples possibly a subculture and identification test has to be made from all dilution tubes. With the membrane filtration method it is possible to determine total coliforms and faecal coliforms with a simplified procedure: counting the lactose-positive colonies after incubation of Endo agar with variable temperatures. There may be a high number of suspect colonies. If it were necessary to identify these colonies the membrane filter method because of the workload would not be interesting for water monitoring of bathing places. According to article 6, paragraph 5 of the Guideline it is allowed to use other methods than those described: "Reference methods of analysis for the parameters concerned are set out in the ANNEX. Laboratories which employ other methods must ensure, that the results obtained are equivalent or comparable to those specified in the ANNEX".

Necessarily the idea arises whether it is possible to gain the same or at least nearly the same reliability of the results with regard to the hygienic evaluation of surface waters with a simple procedure. In this connection the MPN-method with enrichment in BRILA-MUG-broth (= FLUOROCULT-BRILA-broth) and exclusive evaluation of gas production and fluorescence as well as additional indole production are of special interest. The biochemical reactions mentioned are carried out in the dilution tubes as so-called "one-tube-test". The simplified procedures of examination are represented in Table III. With identical water samples from different surface waters (coastal waters and inland waters) the results of a one-tube-test were compared with the results from a MPN-method with enrichment in lactose (without supplements) and identification of the positive dilution tubes /3/. As for the procedure see Table IV.

Table II

Methods of analysis for total coliforms and faecal coliforms according to the
E.C. Directive for bathing water

Microbiological parameters	Fermentation in multiple tubes. Subculturing of the positive tubes on a confirmation medium. Count according to MPN (most probable number)
1. Total coliforms	or
2. Faecal coliforms	membrane filtration and culture on a appropriate medium such as Tergitol lactose agar, Endo agar, 0.4% Teepol broth, sub-culturing and identification of the suspect colonies
In the case of 1 and 2, the incubation temperature is variable according to whether total or faecal coliforms are being investigated	

Table III

Determination of total coliforms and faecal coliforms with one-tube-test

-
- 3 times each 1.0 - 0.1 - 0.01 ml of the water sample in BRILA-MUG-broth
 - Incubation at 36 °C and 24/44 h
 - Gas production (positive)
 - = Total coliforms (TC)
 - Fluorescence (positive) and indole (positive)
 - = Faecal coliforms (FC)
 - Counting according to MPN (McCrary)
-

Results and discussion

Comparison of the MPN-indices for faecalcoliforms (FC) with the indices for Escherichia coli after biochemical identification (EC) shows — as expected — that values obtained by the one-tube-test tend to be higher than with biochemical identification. The differences may be partly explained by the fact that in the subculture — as usually — only one colony is examined. In surface waters samples there are mixed cultures. Comparison of tests with one and three colonies shows very clearly that the examination of several colonies has a distinct effect on final results (Table 5). After enrichment with lactose broth and subculture according to the quoted identification schedule false negative results occurred relatively frequently, if we made further tests with an extended identification (API 20/20E). This may be an additional explanation /4/.

Table IVDetermination of coliform bacteria and *E. coli* with enrichment, subculture and identification

-
- 3 times each 1.0 - 0.1 - 0.01 ml of the water sample in lactose broth
 - Incubation at 36 °C and 24/44 h
 - Gas production (positive)
 - Subculture on Endo agar
 - Identification: Cx-test
Lactose 37 °C
Indole
Glucose 44 °C
Citrate agar
 - Counting according to MPN (McCrary)
-

Identification pattern for *E. coli* and coliform bacteria

	<u><i>E. coli</i></u> EC	coliform bact. Cf	negative
Cx-test	-	-	+/-
Lactose 37 °C	+	+	+/-
Indole	+	+/-	+/-
Glucose 44 °C	+	Ø	+/-
Citrate agar	Ø	+/-	+/-

Table VFluorescence- and indole-positive dilution tubes after enrichment with BRTLA-MUG-Broth and identification of one resp. three colonies from samples of the Baltic Sea

	n	Parameter	n _x	(%)
1 colony	243	coliform bacteria	222	(91)
		<u><i>E. coli</i></u>	194	(80)
		negative	21	(9)
3 colonies	344	coliform bacteria	332	(97)
		<u><i>E. coli</i></u>	307	(89)
		negative	12	(3)

Comparison of MPN-indices for total coliforms (TC) after exceptional examination of gas production with the indices for coliform bacteria (Cf) after subculture and identification shows a similarity as seen at the in-

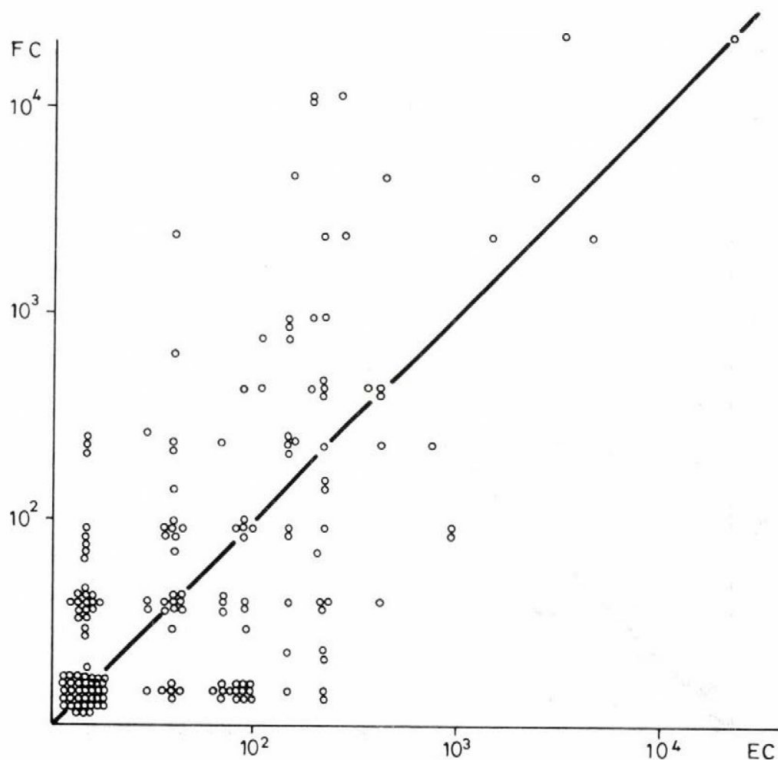


Fig. 1. Comparison of the MPN-indices/100 ml of the faecal coliforms FC (fluorescence in BRILA-MUG-Broth) with *E. coli* EC (lactose-broth and subculture with biochemical identification)

dices for faecal coliform bacteria. The results for the one-tube-test were clearly higher.

During the hot months of the summer season last year and also this year we found especially in water samples from lakes very high values for total coliforms and in the same sample extremely low values for faecal coliforms. In many cases we observed a high contamination with nutrients as well nitrogen substances and phosphates as organic matters. From several samples containing more than ten times of coliforms than faecal coliforms, we investigated the suspect colonies with an extended identification scheme (API-20/20E). The results are summarized in Table VI and listed according to bacterial species in Table VII.

Apparently the high values can not only be explained by a high contamination with bacteria from the family of *Enterobacteriaceae*. Very often we found bacteria belonging to the genus *Aeromonas* alone or together with

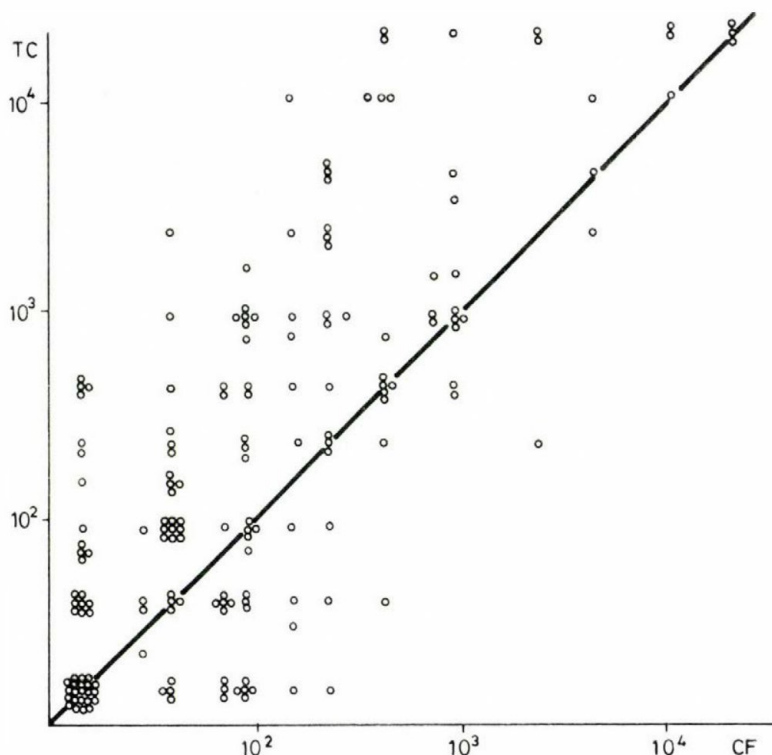


Fig. 2. Comparison of the MPN-indices/100 ml of the total coliforms TC (gas in BRILA-MUG-Broth) with coliform bacteria CF (lactose-broth and subculture with biochemical identification)

Enterobacteriaceae. I.e., we isolated bacteria which in water hygienic measures — for instance in tap water — are usually not included in the

Table VI

Identification of total coliforms from water samples
with a big ratio of total coliforms ($TC > 10 \times FC$ / $n = 42$)

	n	(%)
<u>Aeromonas</u> spp.	18	(43)
<u>Enterobacteriaceae</u>	4	(10)
<u>Aeromonas</u> spp. + <u>Enterobacteriaceae</u>	15	(36)
<u>Aeromonas</u> spp. + other	3	(7)
<u>Enterobacteriaceae</u> + other	2	(4)

Table VII

Frequency of *Aeromonas* spp., Enterobacteriaceae and other bacteria in water samples with a big ratio of total coliforms (n = number of isolated colonies)

	n	(%)	Σn	(%)
<u><i>Aeromonas hydrophila/caviae</i></u>	26	(36.1)		
<u><i>A. sobriai</i></u>	17	(23.6)		
<u><i>Aeromonas</i> spp.</u>	3	(4.2)	46	(63.9)
<u><i>Enterobacter</i> spp.</u>	8	(11.1)		
<u><i>Hafnia</i> spp.</u>	6	(8.3)		
<u><i>Citobacter</i> spp.</u>	3	(4.2)		
<u><i>Serratia</i> spp.</u>	3	(4.2)		
<u><i>Escherichia</i> spp.</u>	2	(2.7)	22	(30.5)
<u><i>Pseudomonas</i> spp.</u>	3	(4.2)		
<u><i>Acinetobacter</i> spp.</u>	1	(1.4)	4	(5.6)
	72	(100.0)	72	(100.0)

scheme of examination (cx-positive) and not included in evaluation of water samples.

According to the present conception representatives of the genus *Aeromonas* have to be considered as facultative pathogenic agents /4/. This is correct in those cases, where they occur in high concentrations. *Aeromonas* spp. are ubiquitous in surface waters and can grow under certain circumstances at relative low temperatures. Moreover, a high contamination with *Aeromonas* indicates a highly polluted surface water with a high degree of eutrophication. This is an important additional criterion for the evaluation of bathing waters, even when a direct dependence of faecal contamination does not exist in every case. The degree of an up-to-date faecal contamination can be estimated by the pollution with faecal coliforms.

Conclusion

The one-tube-test is of equal value to a method with subculture and biochemical identification. The test needs only a minimum of material and laboratory staff. Differences between this test and other more extensive tests with several biochemical identification steps are negligible. The work load for bathing water monitoring would not be justified in this case.

Caused by the occurrence of aeromonas, the values for total coliforms compared with a determination with biochemical identification are higher in some cases. This is not contrary to the E.C. Guideline. According to the Guideline the members are allowed to assess other criteria for particular parameters, provided that the quality of bathing waters becomes not worse. According to article 7, paragraph 2 of the Guideline: "Member States may at any time fix more stringent values for bathing water than those laid down in this directive".

An ad hoc-working-group of the coastal countries of the Federal Republic of Germany has recommended the one-tube-test with BRILA-MUG-broth and enumeration by MPN-tables for the determination of total and faecal coliforms according to the E.C. Guideline for bathing water. Meanwhile this method of analysis is part of decrees or laws in some countries of the FRG and has also been formerly applied in the coastal countries of the new FRG.

In revising the methods, the following points are to be especially observed: (i) the method must be handy and economical; (ii) the results must be reproducible and comparable with other results; (iii) the nutrient media should be standardized and their quality should be controlled by users.

The BRILA-MUG-broth shows to be sensitive to different temperatures during the production in the laboratory. Depending on sterilization temperature and time we see various inhibitory effects /5/. For this reason the recommendations of the ad hoc-working-group contains a notice that the instruction concerning the production of the broth has to be exactly observed.

REFERENCES

1. Havemeister, G.: *Öff Gesundh-Wes* 51, 423 (1989).
2. Council Directive of 8th Dec. 1975 concerning the quality of bathing water (76/160/EEC), Official Journal of European Communities, Nr. L 31/1 - 7.
3. Havemeister, G., Aleksic, S., Bockemühl, J., Heinemeyer, E. A., Miller, H. E., von Pritzbuer, E.: *Zentralbl Hyg* 191, 523 (1991).
4. Mascher, F.: *Hyg Med* 15, 480 (1990).
5. Müller, H. E., Aleksic, S., Bockemühl, J., Havemeister, G., Heinemeyer, E. A., von Pritzbuer, E.: *Zentralbl Hyg* 189, 543 (1990).

COMPARISON OF CHROMOGENIC AND FLUOROGENIC SUBSTANCES
FOR DIFFERENTIATION OF COLIFORMS AND ESCHERICHIA COLI IN
SOFT CHEESE

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(Received September 24, 1991)

In food hygiene the differentiation of Escherichia coli and coliforms as index resp. indicator organisms is very important as a basis for the assessment of good manufacturing practice (gmp). Using a fluorogenic (MUG) and chromogenic (X-gal) substrate the laborious methods have become more simple and reliable. A new German DIN standard method (10.183, part 3) was used as a base to examine 200 soft cheese samples for coliforms and Escherichia coli comparing different media, parameters and incubation times. It could be shown that an incubation of 48 h is absolutely necessary for E. coli and coliforms in both media, that X-gal is a quicker and more sensitive parameter for total coliforms than gas production and that the combination of fluorescence and indole is slightly superior to fluorescence and gas for the identification of E. coli. To sum up, the new fluorescence principle, integrated in new standard methods will be an excellent tool to simplify the differentiation of E. coli and coliforms in food hygiene. Additionally, X-gal as a chromogenic substrate for all coliforms, including E. coli, may be integrated in further standard methods.

In food hygiene coliforms since decades are important as marker organisms: as indicators for good manufacturing practice (gmp), — interpreted by the coliforms — or as index organisms for potential faecal contaminations, — interpreted separately by E. coli. Until now the differentiation of E. coli within the group of coliforms was laborious and/or unsatisfactory. By the detection of a fluorogenic substrate — 4-methylumbelliferyl-beta-D-glucuronide (MUG) — which is highly specific for E. coli within the group of coliforms, a very simple and reliable method is available to distinguish these two groups of microorganisms /1-9/. Therefore, national and international standards were necessary and are available now (DIN, IDF) to simplify the differentiation of these kinds of organisms. Additionally, a new substance was found which should be specific for the total coliform group (5-brom-4-

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-chlor-3-indolyl-beta-D-galactopyranosid /X-gal/) used in liquid and solid media according to Manafi and different coworkers /10-12/.

The principle of these two substrates is as follows: (1) MUG is metabolized to methylumbelliferon within the group of coliforms by the glucuronidase of E. coli and yields a bright blue fluorescence in liquid and on solid media. The specificity for E. coli is far more than 90%. (2) X-gal is metabolized by all coliforms and yields a blue-greenish colour in broth and light blue colonies on solid media. So far it could be a substitution for gas production.

In former investigations some years ago we examined as one of the first ones in Germany the reliability of the MUG-test /4/. The solid and liquid media, however, were prepared by ourselves, because commercially no medium ready to use was available at it is today. Pure cultures of E. coli and other coliforms were compared at 30 °C and 44.5 °C in liquid and on solid media. The temperature of 44.5 °C till now is used for selection of the so-called faecal, presumptive or thermotrophic coliforms. As parameters we used fluorescence, gas and indole production. It could be shown that fluorescence and indole are much more reliable than gas production, especially at 44.5 °C. On the basis of these and other results from different laboratories a standard in Germany was developed by DIN, which is nearly ready to be published (DIN 10183, part 3). In this standard coliforms and E. coli are defined as follows: (i) coliforms are bacteria which metabolize lactose and produce gas; (ii) bacteria which metabolize MUG to fluorescent methylumbelliferon and produce indole, belong to E. coli. (E. coli identified by this method correctly are called "presumptive E. coli".)

Materials and methods

Because new media and a lot of experience are available in the meantime the mentioned DIN-standard should be tested using 200 soft cheeses of German origin as sample material. The media used and the examined parameters are shown in Fig. 1. The preparation and examination of cheese samples is shown by a flow diagram (Fig. 2).

Results and discussion

For this examination no conventional control method was used as it should be normally, because the aim mainly was to (i) study the feasibility of the mentioned DIN-standard method; (ii) compare the different incubation times of 24 and 48 h; (iii) compare the reliability and the relation of the

LST - containing MUG and L-tryptophan	Fluorocult-Lauryl-Sulfate Broth (Merck Nr. 12588)
gas production	- <u>Coliforms</u>
gas production	
fluorescence	- <u>E. coli</u>
indol production	
LMX like LST containing additionally X-gal	X-gal (Merck Nr. 24655)
(gas production)* and blue color	- <u>Coliforms</u>
(gas production)*, blue color	
fluorescence, indol production	- <u>E. coli</u>
EMX, solid medium containing X-gal	Fluorocult ECD Agar (Merck Nr. 4038)
blue colonies	- <u>Coliforms</u>
blue and fluorescent colonies	- <u>E. coli</u>

*Durham-tubes may be used additionally in LMX broth as a further confirmation test.

Fig. 1. Media and parameters

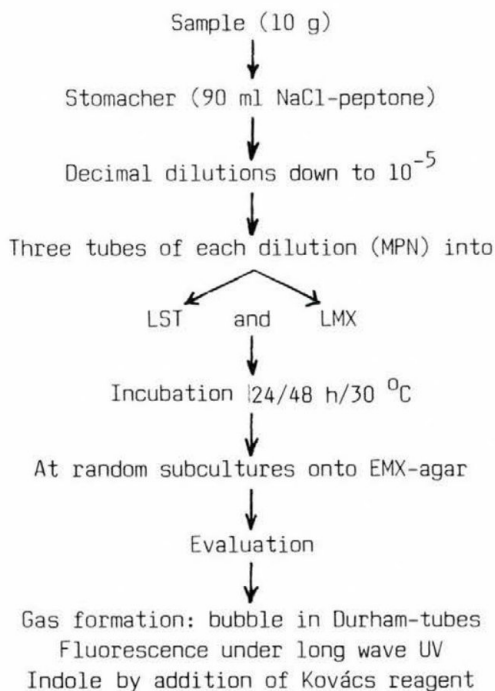


Fig. 2. Preparation and examination of cheese samples

single parameters; (iv) find out the most sensitive parameter and, at least; (v) have some new data about coliforms and E. coli regarding to the new EEC standard for soft cheese with a maximum of 10^5 /g for coliforms and 10^3 for presumptive E. coli. Some few results out of the lot of data we have got will be demonstrated in the following Tables and Figures. In Table I the classification of coliforms and E. coli is based on the definition of the German DIN standard. Additionally, for E. coli the alternative definition "fluorescence + gas" was examined, as it was proposed in the previous draft. The officially used LST-MUG broth is compared to the brand new LMX-MUG broth which shows fluorescence for E. coli and a blue-green colour for E. coli and the other coliforms as a substitution for gas production. From Table I the following conclusions can be made (1) Incubation of 48 h is superior to 24 h and absolutely necessary. This is true for coliforms and E. coli in both media. (2) Blueing in LMX-broth seems to be superior to and quicker than gas production in LST-MUG for identification of coliforms, however, depending on

Table I

Classification of 40 groups of soft cheese (5 of each lot) based on different incubation times (24 h, 48 h) at 30 °C, different media and parameters

Identification - based on	n	Ø	1-2	log 10 MPN/g					
				2-3	3-4	4-5	5-6	6-7	
LST-MUG									
Coliforms	24	8	9	7	5	2	6	3	
- gas	48	3	1	4	11	8	8	5	
<u>E. coli</u>	24	25	8	4	1	1	1	0	
- fluorescence + - gas	48	11	6	11	7	4	0	1	
<u>E. coli</u>	24	20	12	4	3	0	0	1	
- fluorescence + - indole	48	11	8	10	9	1	0	1	
LMX-MUG									
Coliforms	24	5	2	14	10	4	2	3	
- blueing	48	3	2	2	10	14	5	4	
<u>E. coli</u>	24	25	7	6	0	1	0	1	
- fluorescence + - blueing	48	10	6	10	10	2	1	1	
<u>E. coli</u>	24	24	7	5	3	0	0	1	
- fluorescence + - indole	48	11	7	8	12	1	0	1	

the count of bacteria. (3) Concerning the definition for E. coli the combination of fluorescence/indole seems to be more sensitive than fluorescence/gas. A nice correlation between gas production in LST-MUG and blueing in LMX-MUG as a base for the identification of coliforms is shown in Fig. 3. So far, obviously, this new parameter X-gal can be used instead of gas production. The advantage may be to omit the old fashioned Durham tubes which were used for decades till now.

From Table II in a few figures the following facts may be seen again (1) Incubation for 48 h is absolutely superior to 24 h in both media. (2) Fluorescence/gas and fluorescence/indole are nearly identical in both media. (3) Blueing in LMX seems to be more rapid and sensitive than gas production in LST for the identification of coliforms. (4) For E. coli the combination of fluorescence and indole is slightly more reliable than fluorescence and gas production, especially after 24 h incubation.

In Fig. 4 a cumulative frequency distribution of our findings in 200 samples of soft cheeses is shown. Only about 60% of 200 soft cheese samples

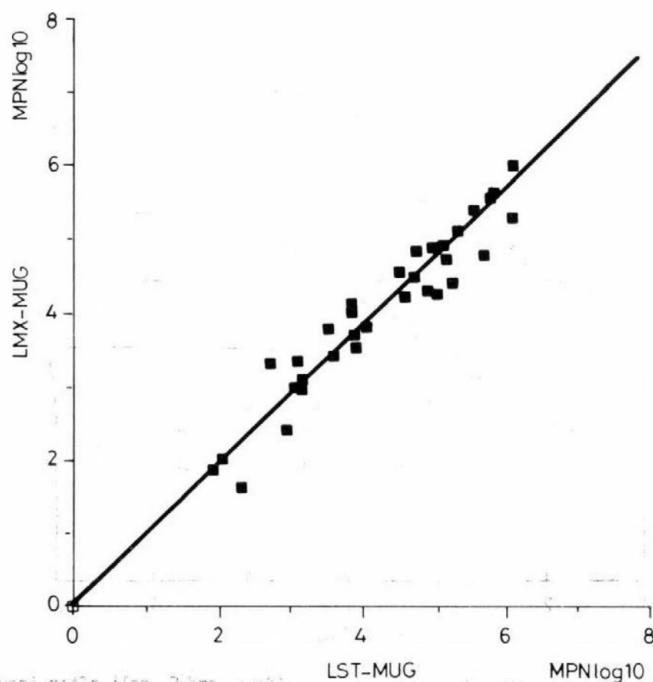


Fig. 3. Correlation of gas production in LST-MUG and blueing in LMX-MUG (30 °C/48 h)

Table II

Comparison of different parameters for the identification of coliforms (G) and *E. coli* (F, FG, FI) depending on different media and incubation times (n = 200; percentages)

Medium Incubation	G	F	FG	FI
LST-broth				
24 h/30 °C	64	29	21	25
48 h/30 °C	91	61	59	56
LMX-broth	B	F	FB	FI
24 h/30 °C	77	28	23	24
48 h/30 °C	88	62	60	57

G = gas; F = fluorescence; I = indole; B = blueing

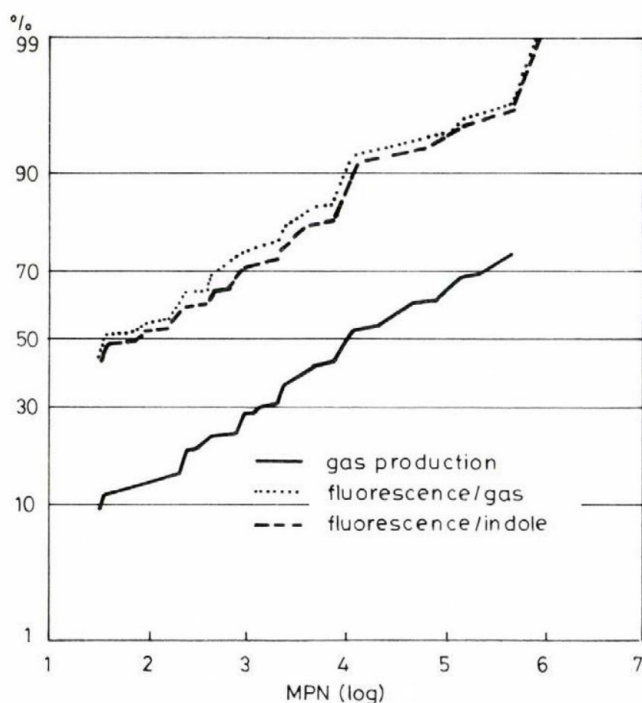


Fig. 4. Cumulative frequency distribution of coliforms and *E. coli* after incubation in LST-MUG (30 °C/48 h).

had few than 10^5 coliforms/g and about 70% few than 10^3 /g of E. coli -- according to the EEC standard. However, this is for products before delivery from the manufacturer and not -- as we did -- from retail stores. And once more it can be seen that the definition for E. coli by fluorescence and gas or indole is nearly equal.

To sum up, the new fluorescence principle, integrated in new standard methods will be an excellent tool to simplify the differentiation of E. coli and coliforms in food hygiene. Additionally, X-gal as a chromogenic substrate for all coliforms, including E. coli may be integrated in a further standard method.

REFERENCES

1. Alvarez, R. J.: Journal of Food Science 49, 1186 (1984).
2. Feng, P. C. S., Hartman, P. A.: Applied and Environmental Microbiology 43, 1320 (1982).
3. Glaeser, H., Hangst, E.: Deutsche Molkerei-Zeitung 36, 1170 (1986).
4. Hahn, G.: Milchwissenschaft 42, 434 (1987).
5. Hill, W. E., Ferreira, J. L., Payne, W. L., Jones, V. M.: Applied and Environmental Microbiology 49, 1374 (1985).
6. Moberg, L. J.: Applied and Environmental Microbiology 50, 1383 (1985).
7. Petzel, J. P., Hartman, P. A.: Applied and Environmental Microbiology 49, 925 (1985).
8. Robinson, B. J.: Applied and Environmental Microbiology 48, 285 (1984).
9. Trepeta, R. W., Edberg, S. C.: Journal of Clinical Microbiology 19, 172 (1984).
10. Manafi, M., Kneifel, W.: Zentralb Hyg 189, 225 (1989).
11. Manafi, M., Kneifel, W.: Applied Fluorescence Technology 6, 11 (1989).
12. Manafi, M., Kneifel, W., Bascomb, S.: Microbiological Review 55, 3 (1991).

RAPID IDENTIFICATION OF ESCHERICHIA COLI FROM URINE BY USING FLUOROCULT MEDIA

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(Received September 24, 1991)

For rapid identification of Escherichia coli, we evaluated Fluorocult MacConkey Agar, Fluorocult Laurylsulfate Broth and Bactident E. coli, which are incorporating fluorogenic substrate, MUG (4-methylumbelliferyl-beta-D-Glucuronide) that specifically reacts with E. coli. To assess the specificity and sensitivity of Fluorocult MacConkey Agar and Laurylsulfate Broth, beta-D-glucuronidase; beta-GUR activities of 264 strains from urine including 72 of E. coli were investigated. For both media, sensitivity was 92% and specificity was 100%. When there was 10^8 c.f.u./ml of E. coli in urine specimen, incubation times required for positive fluorescence by Fluorocult MacConkey Agar, Laurylsulfate Broth, and Bactident E. coli were 8 h, 4 h and 15 min, respectively. Influence of drugs in urine to fluorescence reaction was not observed.

It is said that Escherichia coli is the cause of approximately 80% of acute cystitis. In these cases, there are 10^8 c.f.u. per ml of E. coli in the urine specimens. Mitsui Memorial Hospital where I am working for, is located in downtown Tokyo, and has about 500 beds. The number of outpatients are about 1400 per day on average. The incidence of major organisms isolated from outpatients' and inpatients' urine at Mitsui Memorial Hospital in 1990 is shown in Fig. 1. Out of 1486 urine specimens of outpatients, 926 contained not less than 10^4 c.f.u./ml, 168 not more than 10^3 c.f.u./ml, and 392 specimens were negative. From urine specimens of outpatients, 2038 strains were isolated, and 14.1% of them were Escherichia coli. On the other hand, in 2731 urine specimens of inpatients, 948 specimens contained not less than 10^4 c.f.u./ml, 2051 strains were isolated, and 8.6% of them were E. coli. In both outpatients and inpatients, E. coli was the most frequently isolated.

The incidence of E. coli isolated from urine at Mitsui Memorial Hospital in the last 7 years is presented in Fig. 2. In 1987, a slight decrease was found in both outpatients and inpatients. However, remarkable changes

were not found in the other years. As shown in Fig. 2, about 300 strains of E. coli were isolated from outpatients, and its percentage was 19%. From inpatients, more than 100 strains of E. coli were isolated, and its percentage was 4-6%. From these results, it is clear that the rapid diagnosis of E. coli is very important.

For reference, Table I indicates the products incorporating MUG for detection of E. coli, recently marketed in Japan. Many MUG products other than Fluorocult are available, but they are not popular in Japan yet.

Materials and methods

Strains. For evaluation of Fluorocult, 264 strains, recently isolated from urine specimens at our laboratory, were used (Table II). And also 57 urine specimens, in which E. coli had been detected, were tested directly.

Test method of Fluorocult media to evaluate sensitivity and specificity. Each test strain and urine specimen was inoculated in both Fluorocult MacConkey Agar and Fluorocult Laurylsulfate Broth. After incubation at 37 °C overnight, blue-purple fluorescence was observed under 366 nm UV lamp. Biotest 1 (Eiken Co., Japan) was used for routine identification of Enterobacteriaceae, and Rapid 20E (API bioMérieux, France) was used for the rapid identification.

Test method for the counts of E. coli and incubation time required. The suspension of E. coli was diluted to 10^8 - 10^5 c.f.u./ml, and inoculated to both Fluorocult MacConkey Agar and Laurylsulfate Broth. The incubation time required for positive fluorescence was determined.

Test method for quantities of MUG and incubation time required (Fig. 3). Fluorocult Laurylsulfate Broth contains 0.1 g per litre of MUG originally. MUG was further added to make double and triple concentrations. Incubation time for positive fluorescence was determined for each MUG concentration.

Results

Sensitivity and specificity. Table III shows the test results of sensitivities and specificities of Fluorocult MacConkey Agar and Laurylsulfate

Table I
MUG products for Escherichia coli

	Product name	Reaction	Manufacture—Supplier
Culture media	Fluorocult MUG shot CPS ID	MacConkey + β -GUR β -GUR, TDA, Esculin	Merck-Aska GIBCO bioMérieux-Syntek
Dip slide	Cult-Dip Plus MD-colidip Urine ID	β -GUR	Merck-Aska Ames-Miles Sankyo bioMérieux-Syntek
Test kits	Bactident E. coli MUG Disk	β -GUR, IND	Merck-Aska remel-Cosmobio

Table II
Tested strains isolated from urine

Enterobacteriaceae	121 strains	Glucose-nonfermenting Gram-negative rods	36 strains
<i>Escherichia coli</i>	72	<i>Pseudomonas aeruginosa</i>	10
<i>Citrobacter freundii</i>	6	Others	26
<i>Klebsiella pneumoniae</i>	7		
<i>K. oxytoca</i>	4	Gram-positive cocci	84 strains
<i>Enterobacter cloacae</i>	9	<i>Staphylococcus aureus</i>	21
<i>E. aerogenes</i>	6	Coagulase negative staphylococci	12
<i>Serratia marcescens</i>	6	<i>Streptococcus agalactiae</i>	29
<i>Proteus vulgaris</i>	2	<i>Enterococcus faecalis</i>	18
<i>P. mirabilis</i>	3	Others	4
<i>Morganella morganii</i>	4		
<i>Providencia rettgeri</i>	1	Yeast	23 strains
<i>Salmonella</i> spp.	1	Total	264 strains

Broth by using clinical isolates. In both media, 66 were positive and 6 were negative out of 72 strains of *E. coli*. The sensitivity was 92%, and the specificity was 100%. Although out of 29 strains of *Streptococcus agalactiae*, 24 (84%) were fluorescence-positive, they could be easily distinguished by the Gram-staining and the colony size. On the other hand, 53 out of 57 urine specimens were fluorescence-positive. The bacterial counts of 4 fluores-

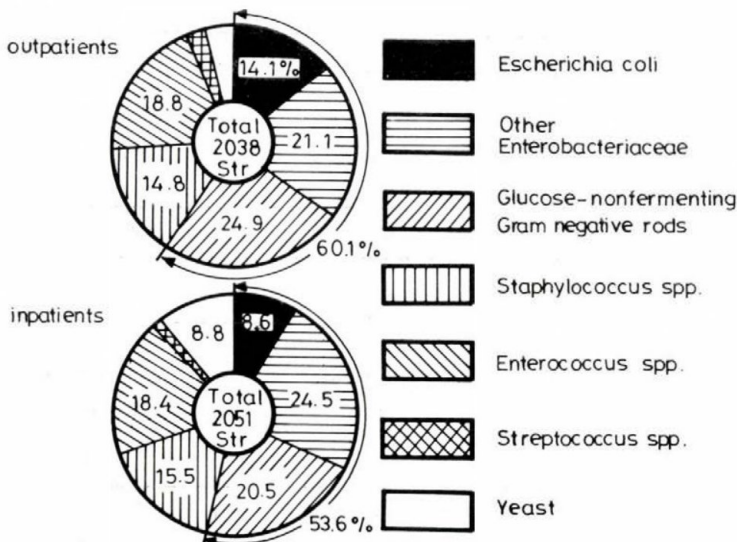


Fig. 1. Incidence of major organisms isolated from urine at Mitsui Memorial Hospital in 1990. Comparison between outpatients and inpatients

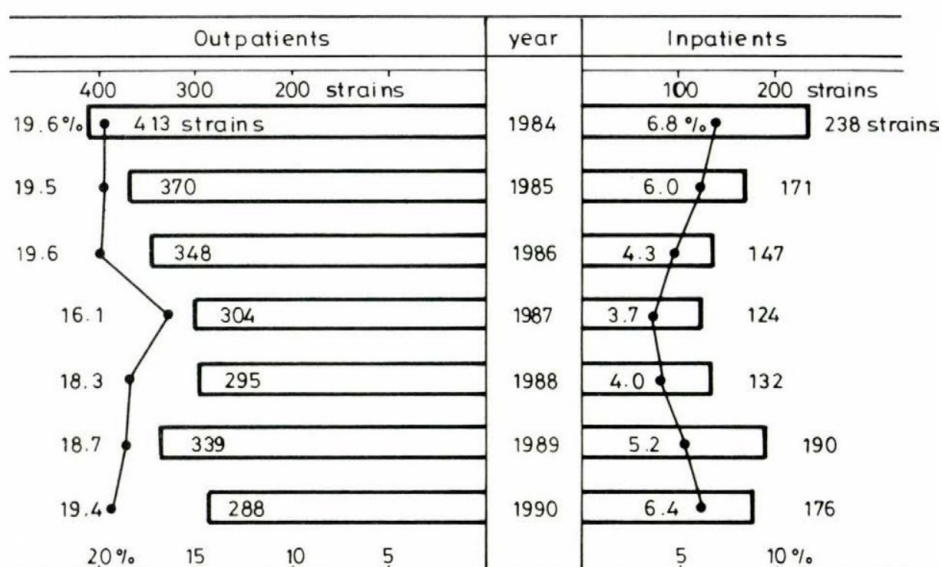


Fig. 2. Incidence of *E. coli* isolated from urine at Mitsui Memorial Hospital in the last 7 years

cence-negative specimens were checked. Two specimens contained 10^8 c.f.u./ml, and the remaining 2 specimens 10^4 c.f.u./ml.

The counts of *E. coli* and incubation time required for positive fluorescence by using Fluorocult MacConkey Agar are shown in Fig. 4. When counts

Quantities of MUG and incubation time required

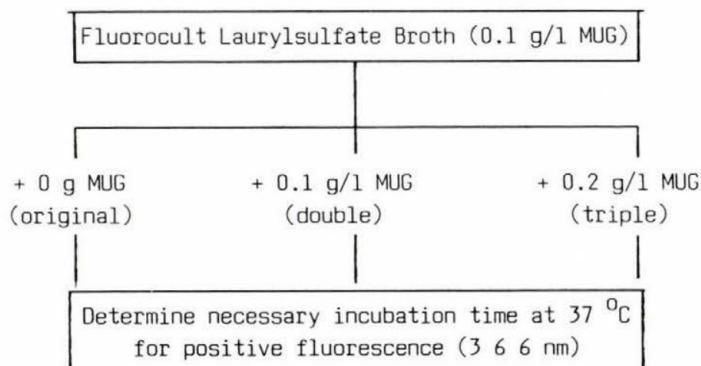


Fig. 3. Test methods

Table III
Sensitivity and specificity for β -GUR of urine

	No. of strains	Fluorescence			
			positive		negative
<u>E. coli</u>	72	A	66	B	6
Other Gram-negative rods	85	C	0	D	85

$$\text{Sensitivity} = \frac{A}{A+B} \times 100 = \frac{66}{72} \times 100 = 92\%$$

$$\text{Specificity} = \frac{D}{C+D} \times 100 = \frac{85}{85} \times 100 = 100\%$$

of E. coli were 10^8 c.f.u./ml, it took 8 h until fluorescence became positive.

The counts of E. coli and incubation time required for positive fluorescence by using Fluorocult Laurylsulfate Broth are set out in Fig. 5. When the counts of E. coli were 10^8 c.f.u./ml, the incubation was reduced to 4 h. As we expected, the incubation time required for Fluorocult Laurylsulfate Broth was shorter than that of Fluorocult MacConkey Agar. The similar results were obtained when urine specimens were inoculated directly.

Quantities of MUG and incubation time required. Double and triple concentrations of MUG (0.2 g/l and 0.3 g/l) did not shorten the incubation

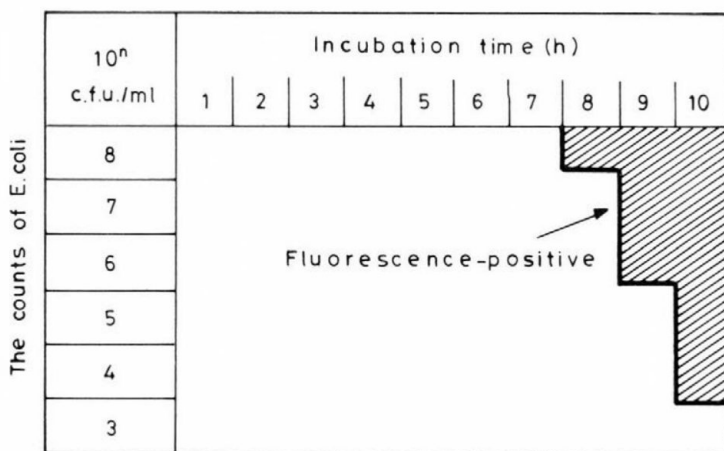


Fig. 4. The counts of E. coli and the incubation time required for positive fluorescence by using Fluorocult MacConkey Agar

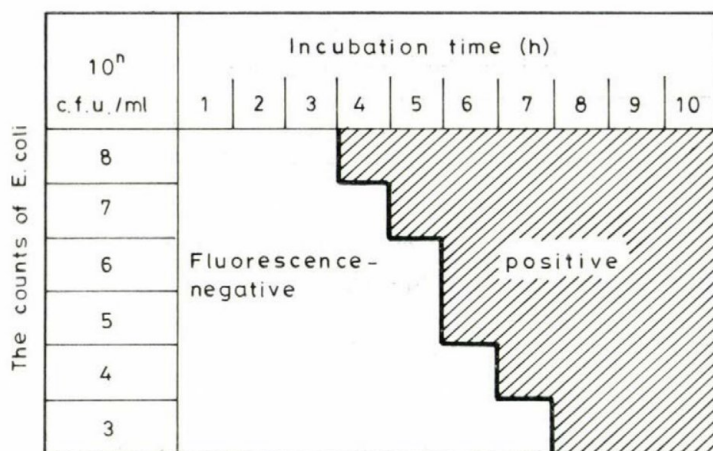


Fig. 5. The counts of *E. coli* and the incubation time required for positive fluorescence by using Fluorocult Laurylsulfate Broth

time for positive fluorescence, compared to the original quantity of MUG.

Influence of drugs to fluorescence. As mentioned before, 6 out of 72 strains of *E. coli* were fluorescence negative. Since false-negative due to

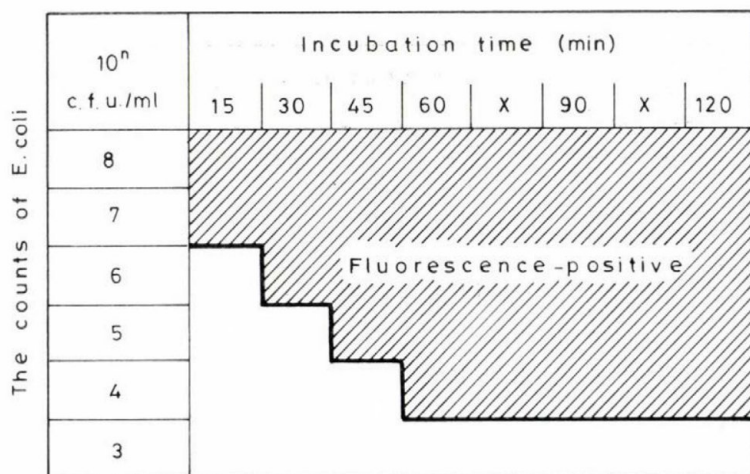


Fig. 6. The counts of *E. coli* and the incubation time required for positive fluorescence by using Bactident *E. coli*

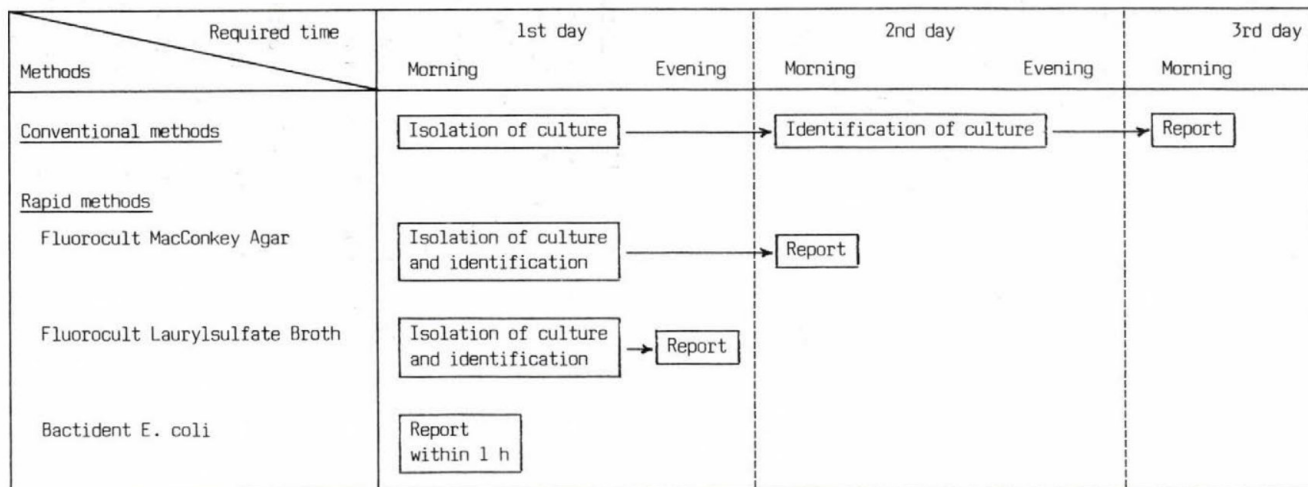


Fig. 7. Comparison of reporting time required for urine screening in case *E. coli* is suspected by Gram-staining

the influence of drugs in urine was suspected, we repeated the isolation culture for the 6 fluorescence negative strains 3 times. However, all of the strains were still negative. Since false-positivity due to drugs in urine was also suspected, we checked the fluorescence reaction for 300 urine specimens which were confirmed sterile, but containing drugs. However, no false-positive due to drugs was observed.

The counts of E. coli and incubation time required for positive fluorescence by using Bactident E. coli is shown in Fig. 6. By using Bactident E. coli, we found that the incubation time for positive fluorescence and indole reaction could be shortened. When there were 10^{7-8} c.f.u./ml and 10^6 c.f.u./ml of E. coli, it took only 15 min and 30 min, respectively. When there was only 10^5 c.f.u./ml, it took 45 min. When untreated urine specimen or centrifuged sediment was checked by Bactident E. coli, we could identify E. coli within 1 h. From this result, we think Bactident E. coli is also useful for rapid identification of E. coli, in case the acute cystitis is suspected with the symptoms of the patients, and E. coli is suspected by Gram-staining of urine specimens.

Conclusions

(1) By using Fluorocult media, isolation and identification of E. coli can be done by one step. (2) Sensitivity was 92% and specificity was 100% for both Fluorocult MacConkey Agar and Laurylsulfate Broth. (3) Requirement of incubation times for positive fluorescence by Fluorocult MacConkey Agar, Laurylsulfate Broth, and Bactident E. coli were: (i) Fluorocult MacConkey Agar, 10^8 c.f.u./ml: 8 h; 10^5 c.f.u./ml: 10 h. (ii) Fluorocult Laurylsulfate Broth, 10^8 c.f.u./ml: 4 h; 10^5 c.f.u./ml: 8 h. (iii) Bactident E. coli, 10^8 c.f.u./ml: 15 min; 10^5 c.f.u./ml: 45 min. An influence of drugs in urine to the fluorescence reaction was not observed.

As to the importance of Fluorocult and Bactident E. coli for urine screening, the following may be concluded. (1) By using Fluorocult and Bactident E. coli, clinical technicians can report the result of urine screening to physicians promptly, whether E. coli is causative bacteria or not (Fig. 7). (2) Since the physicians can select the antibiotics after getting the result of urine screening, they will not have to use strong antibiotics with broad spectrum such as new quinolone agent, in case E. coli is detected from urine specimens. This tendency will be able to prevent the increase of resistant organisms. Furthermore, for the remaining 20% of urinary tract

infection, caused by microorganism other than E. coli, serious clinical consequences of missed diagnoses will be avoided /1-5/.

Acknowledgements. The authors wish to thank JUN OKADA of Kanto Teishin Hospital, Tokyo, Japan for critical review of the manuscript and FUMI SUZUKI of Merck Japan for secretarial assistance.

REFERENCES

1. Trepeta, R. W., Edberg, S. C.: J Clin Microbiol 19, 172 (1984).
2. Delisle, G. J., Ley, A.: J Clin Microbiol 27, 778 (1989).
3. Kilian, M., Bülow, P.: Acta Path Microbiol Scand Sect B 84, 245 (1975).
4. Moberg, L. J.: Food Appl Environ Microbiol 50, 1383 (1985).
5. Heizmann, W. R., Doeller, P. C., Werner, H.: J Microbiol Methods 12, 51 (1990).

BETA-D-GLUCURONIDASE (BDG) ACTIVITY OF GRAM-NEGATIVE BACTERIA

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(Received September 24, 1991)

BDG is an inducible enzyme that is encoded by the uidA gene in Escherichia coli. Genetic sequences of this gene are present in most if not all E. coli strains regardless of the BDG phenotype. Expression of BDG activity can be influenced by lactose-induced catabolite repression or genetic mutations. Salmonella, Shigella and Yersinia strains frequently exhibit positive BDG reaction. BDG activity of strains belonging to genus Edwardsiella, Serratia, Yersinia, Vibrio, Erwinia, Alcaligenes, Acinetobacter, Moraxella, Plesiomonas, Achromobacter, Flavobacterium, Chromobacterium and Pasteurella awaits examination.

Coliform bacteria have been considered indicators of faecal pollution. These bacteria, as a group, can be characterized by production of gas and acid from lactose (Fig. 1) or by coloured colonies on special media (Fig. 2) /1-3/. These criteria are imprecise because some strains of Escherichia species either do not produce gas or produce it only after 48 h. Furthermore, the ability to ferment lactose is a character which can be observed among Gram-negative bacteria other than E. coli, too. In addition, the result of lactose fermentation may be influenced by bacterial interference and by a greater sensitivity of an Escherichia strain to higher incubation temperature and/or selective media used /4/. Detection and enumeration of E. coli strains in food and water instead of coliforms or faecal coliforms

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are more adequate as E. coli strains may always be present in faeces. The classical confirmatory tests which have to be used for the identification of E. coli strains require several days and they are very laborious as seen in Figs 1 and 2. The question is whether the methods which are based on BDG activity of bacterial strains are more exact as well as more economic and whether they present results earlier than the classical procedures? BDG is an inducible enzyme that is encoded by the uidA gene in E. coli /5, 6/. This enzyme cleaves the acyl substrate of the esters of beta-D-glucuronic acid (Fig. 3). For example 4-methyl-umbelliferyl is one of the substrates. This substrate is non-fluorogenic in bound form but becomes fluorogenic after hydrolysis /7/. With the help of this reaction the BDG positive colonies can be identified on a suitable plate or BDG positive culture in a convenient fluid medium.

Materials and methods

For the methods used see reference /4/.

Results and discussion

Taxonomic and diagnostic importance of BDG activity of microorganism has been studied since the 1950s /4/. The results of different authors are summarized in Table I. The enzyme is phenotypically present in 94.0% of E. coli strains in general. We observed 94.8% positivity in case of 327 E. coli "O", "K" and "H" reference strains. Six authors published results between 93.2% and 100.0% /8-13/. In contrast to these, three writers observed less than 90% positivity. Their values were between 65% and 89.4% /14-16/.

Of BDG negative E. coli reference strains, at least 17 strains (10%) out of the 170 "O" serogroup representatives were negative (Table II). Out of the negative serotypes, E. coli O124:K72:H30 which is a well-known enteroinvasive serotype. Five other serotypes may perhaps be in connection with diarrhoeal events (O8:K46:H30, O20ab:K84:H26, O25:K11:H6, O55:K59:H- and O78:K80:H-). The other 11 negative strains are not associated with enteric diseases. Kilian and Bülow /17/ serotyped 94 BDG negative E. coli strains; 25 had O1:K1:H- antigens and 10 isolates O75:K-:H- antigens. Further 30 strains belonged to 25 different serotypes, 22 were rough and the antigenic structure of 7 isolates was unknown. Doyle and Schoeni /18/ ob-

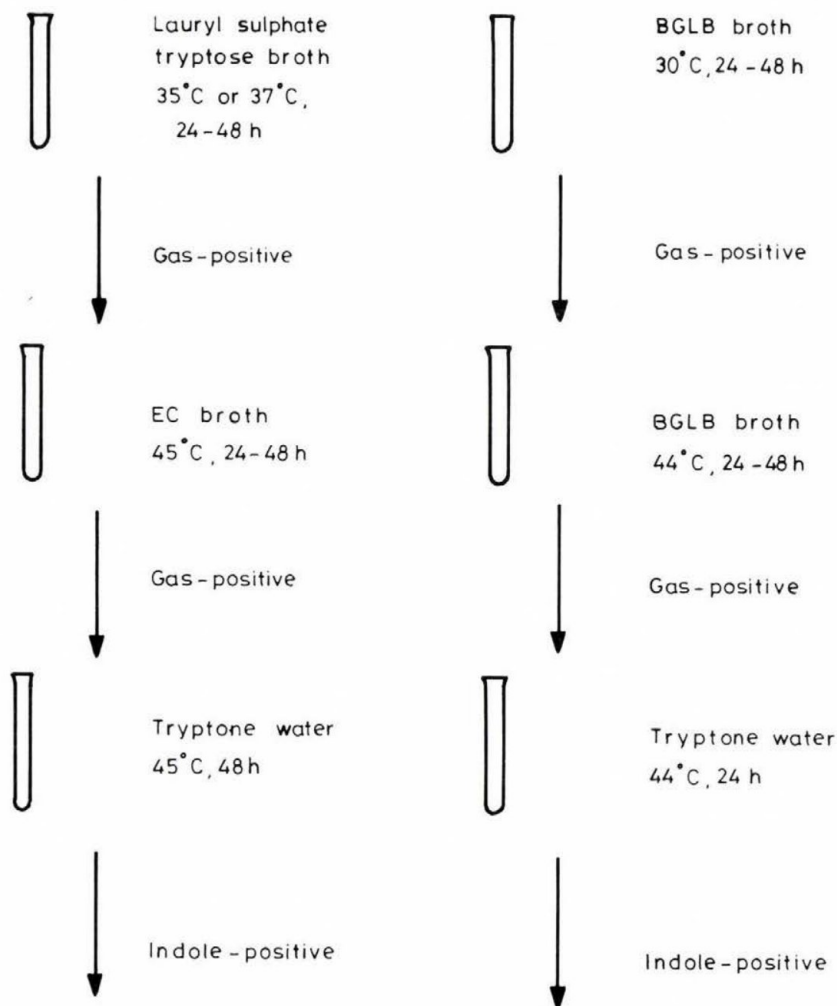
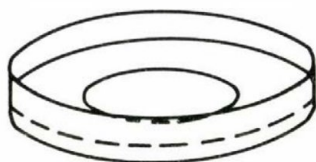


Fig. 1. Detection and enumeration of presumptive *E. coli* by standard most probable number (MPN) tests — ISO 7251 and ISO 4831. Final identification by the ICMSF and the AOAC methods requires another 4-6 days

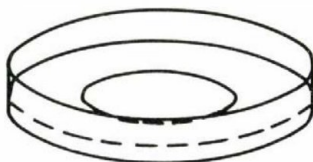
served that enterohaemorrhagic *E. coli* strains O157:H7 were BDG negative. Later this observation was verified by other /4/. Feng et al. /19/ published that three *E. coli* O111:K58 strains and one O1 strain were BDG negative. Bülte and Reuter /8/ also observed one BDG negative ETEC strain. Chang et al. /16/ frequently isolated BDG negative *E. coli* strains from faeces of healthy persons and rats, therefore, they thought that BDG might be a convenient tool for detecting some *E. coli* but it could not be used for the demonstration of faecal coli.



Membrane filter overlaying
minerals modified glutamate
agar for resuscitation
35°C, 4 h



Transfer membrane filter to tryptone
bile agar



Incubation at 44°C, 18-24 h



Indole reaction on the membrane filter

Fig. 2. Detection and enumeration of presumptive *E. coli* by standard direct plating (DP) method - ISO/DP 6391. Final identification by IMViC requires 4 days more

As the frequency of BDG positive and negative *E. coli* serotypes in the same serogroup was not known, we checked 23 serotypes of three serogroups (Table III). Each of the serogroups contained one or more BDG negative serotypes besides the positive ones. Accordingly, BDG positive and negative *E. coli* strains may belong to the same serotype. It is an open question whether BDG activity of an *E. coli* strain may be altered and what kind of factors can influence this character. Colony blots, Southern analyses and polymerase chain reactions showed that part of the genetic sequences of the *uidA* gene was present in most if not all *E. coli* isolates regardless of the BDG phenotype. There is evidence that its expression is affected by lactose-induced catabolite repression. Genetic mutations in the regulatory or structural regions of the genom can also affect *uidA* expression or the production

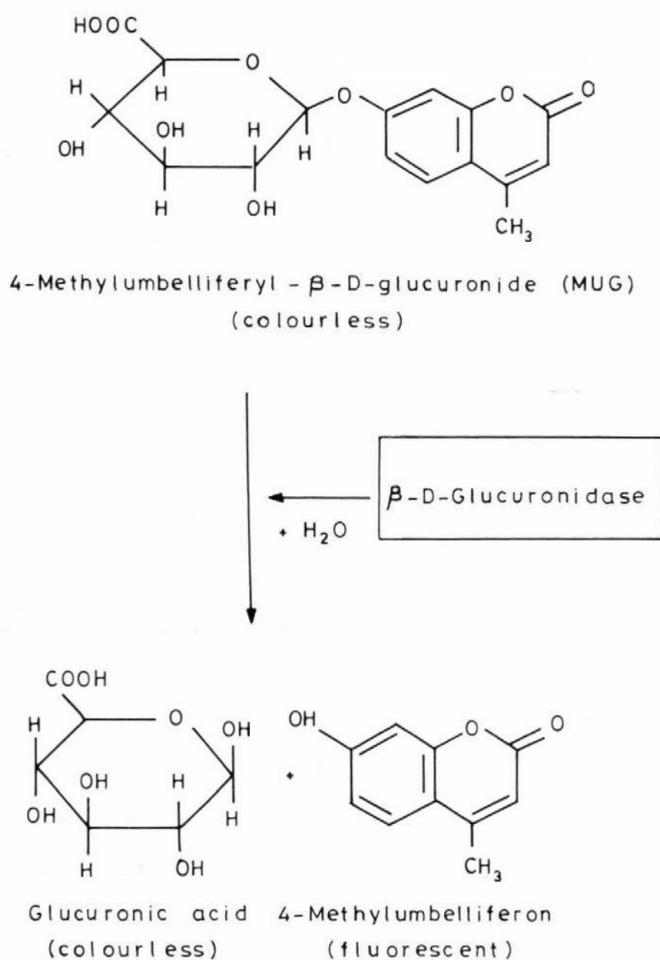


Fig. 3. The effect of beta-D-glucuronidase on MUG

of non-functional enzymes /19/. When the virulence — keratoconjunctivitis-causing capacity — of enteroinvasive *E. coli* and *Shigella* strains was compared with their own BDG activity then it could be assumed that some relationship might exist between these characters. Moreover, Thompson et al. /20/ described a connection between verocytotoxin production and RM-MUG po-

Table I
Beta-D-glucuronidase activity of Gram negative bacteria

Genera	Number of strains	Number of positive strains	Per cent
<u>Citrobacter</u>	193	4	2.0
<u>Edwardsiella</u>	7	-	.
<u>Enterobacter</u>	291	1	0.3
<u>Escherichia</u>	2395	2252	94.0
<u>Hafnia</u>	111	-	0.0
<u>Klebsiella</u>	640	3	0.4
<u>Proteus</u>	854	-	0.0
<u>Salmonella</u>	749	277	36.9
<u>Arizona</u>	69	29	42.0
<u>Serratia</u>	96	3	3.1
<u>Shigella</u>	524	267	50.9
<u>Yersinia</u>	62	6	9.6
<u>Pseudomonas</u>	114	2	1.7
<u>Aeromonas</u>	135	-	0.0
<u>Vibrio</u>	70	-	0.0
<u>Erwinia</u>	14	-	0.0
<u>Alcaligenes</u>	2	-	.
<u>Acinetobacter</u>	2	-	.
<u>Moraxella</u>	1	-	.

Table II
Serotype of beta-D-glucuronidase negative
E. coli reference strains

Serotypes	Association with human diarrhoeal diseases
08:K46:H30	ETEC ?
019b:K.:H7	.
020a,20b:K84:H26	ETEC ?
025:K11:H6	ETEC ?
055:K59:H.	EPEC ?
060:K.:H-	.
063:K.:H-	.
078:K80:H-	ETEC ?
082:K.:H-	.
089:K.:H16	.
091:K.:H-	.
093:K.:H8	.
0108:K.:H10	.
0120:K.:H6	.
0124:K72:H30	EIEC
0136:K78:H-	.
0163:K.:H.19	.
ETEC=Enterotoxigenic	
EPEC=Enteropathogenic	
EIEC=Enteroinvasive	
?=questionable	
.=no data	

Table III
E. coli serogroups including beta-D-glucuronidase positive
and negative serotypes

Serotypes	Number of positive strains	Number of negative strains
O1:K51:H-	1	
O1:K.:HNT		1
O1:K1:HNT	1	
O8:K.:H9	1	
O8:K8:H4	1	
O8:K.:H21	1	
O8:K.:H20	1	
O8:K25:H9	1	
O8:K27:H-	1	
O8:K40:H9	1	
O8:K41:H11	1	
O8:K42:H-	1	
O8:K43:H11	1	
O8:K44:H-	1	
O8:K45:H9	1	
O8:K46:H30		1
O8:K47:H2	1	
O8:K48:H9	1	
O8:K49:H21	1	
O8:K50:H-	1	
O124:K72:H-	3	
O124:K72:HNT		5
O124:K72:H30		1

sitivity of E. coli O157 strains. To get some information we examined two BDG positive virulent E. coli O124:K72:H30 strains. After incubation and repeated passage at an elevated temperature (42 °C) these strains lost their

Table IV
Biochemical properties of Escherichia species

Species	Per cent positive								BDG
	Indole	MR	VP	Cit- rate	Lac- tose	Gas from lactose	H ₂ S	KCN	
<u>E. adecarboxylata</u>	>90	>90	0	<10	>90	>95	<10	>90	-
<u>E. blattae</u>	0	100	0	50	0	0	0	0	?
<u>E. coli</u>	98	99	0	1	95	95	1	3	+
<u>E. coli</u> inactive	80	95	0	1	25	25	1	1	+
<u>E. fergusonii</u>	98	100	0	17	0	0	0	0	-
<u>E. hermannii</u>	99	100	0	1	45	45	0	94	-
<u>E. vulneris</u>	0	100	0	0	15	15	0	15	-

virulence but they kept their BDG positivity. Thus, no strict link was verified between keratoconjunctivitis-causing capacity and BDG activity /21/.

As to biochemical properties of other Escherichia species the results can be seen in Table IV. BDG activity of Escherichia blattae stains has not been examined. In case of Escherichia adecarboxylata, Escherichia fergusonii, Escherichia hermannii and Escherichia vulneris isolates the number of strains examined was too small but they were all negative /11, 20, 22, 23/.

Finally, it was studied whether there was any connection between the properties of late lactose fermentation and BDG activity of E. coli strains. The results were presented in reference /4/. Summarizing, out of 26 late lactose fermenters only 7 strains were BDG negative but 4 strains of the latter originated from the same source. Therefore, it is thought that the result of BDG test is not directly linked to the character of lactose splitting. On the basis of our present knowledge Salmonella, Arizona, Shigella and Yersinia strains frequently show positive BDG reaction, in 36.9%, 42.0%, 50.9% and 9.6%, respectively (Table I). Members of the other genera were practically BDG negative. However, in case of Edwardsiella, Serratia, Yersinia, Vibrio, Erwinia, Alcaligenes, Acinetobacter and Moraxella strains the exact rate of BDG positivity is unknown because the number of the strains examined is small. Also, it would be very necessary to test BDG activity of Plesiomonas, Achromobacter, Flavobacterium, Chromobacterium and Pasteurella strains.

REFERENCES

1. The International Standards Organization (ISO) 4831 (1978).
2. The International Standards Organization (ISO) 7251 (1984).
3. The International Standards Organization (ISO/DIS) 6391 (1985).
4. Ralovich, B., Ibrahim, G. A. M., Fábíán, A., Herpay, M.: Acta Microbiol Hung **38**, 147 (1991).
5. Novel, M., Novel, G.: J Bacteriol **127**, 418 (1976).
6. Blanco, C., Ritzenthaler, P., Mata-Gilsinger, M.: Mol Gen Genet **199**, 101 (1985).
7. Mead, J. A. R., Smith, J. N., Williams, R. T.: Biochem J **61**, 569 (1955).
8. Bülte, M., Reuter, G.: Zentralbl Hyg **188**, 284 (1989).
9. Hartman, P. A.: 5th International Symposium on Rapid Methods and Automation in Microbiology, Florenz, 1987.
10. Feng, P. C. S., Hartman, P. A.: Appl Environ Microbiol **43**, 1320 (1982).
11. Kilian, M., Bülow, P.: Acta Path Microbiol Scand Sect B, **84**, 245 (1976).
12. Massenti, M. F., Scarlata, G., Nastasi, A.: Boll Ist Sieroter Milan **60**, 26 (1981).
13. Hansen, W., Yourassowsky, P. A.: J Clin Microbiol **20**, 1177 (1984).
14. Edberg, S. C., Konnick, C. M.: J Clin Microbiol **24**, 368 (1986).
15. Delisle, G. J., Ley, A.: J Clin Microbiol **27**, 778 (1989).
16. Chang, G. W., Brill, J., Lum, R.: Appl Environ Microbiol **55**, 335 (1989).

17. Kilian, M., Bülow, P.: Acta Path Microbiol Scand Sect B, 87, 271 (1979).
18. Doyle, M. P., Schoeni, J. L.: Appl Environ Microbiol 48, 855 (1984).
19. Feng, P., Lum, R., Chang, G. W.: Appl Environ Microbiol 57, 320 (1991).
20. Thompson, J. S., Hodge, D. S., Borczyk, A. A.: J Clin Microbiol 28, 2165 (1990).
21. Tóth, I., Herpay, M., Ralovich B.: Unpublished data.
22. Heizmann, G., Döller, P. C., Gutbrod, B., Werner, H.: J Clin Microbiol 26, 2682 (1988).
23. Robison, B. J.: Appl Environ Microbiol 48, 285 (1984).

FLUOROGENIC AND CHROMOGENIC SUBSTRATES — A PROMISING TOOL IN MICROBIOLOGY

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(Received September 24, 1991)

During the last few years the use of fluorogenic and chromogenic substrates for rapid and sensitive detection of bacteria has proved to be a powerful alternative to traditional methods. These sophisticated substrates might find widespread application in, for instance, the assay of clinically important enzymes, flow cytometry, and direct epifluorescent filter technique. Specific enzyme detection offers another approach to differential identification and characterization of viable bacteria from a sample. The use of some chromogenic and fluorogenic substrates specific for bacterial enzymes and their applications to microbial identification is reported. Particular emphasis is given to the examination of Escherichia coli and the description of the different techniques as used in routine analysis.

In recent times great emphasis has been placed on the detection, enumeration or quantification of microorganisms using synthetic enzyme substrates or fluorogenic dyes. There are some elegant and sensitive methods using these chemical compounds (Table I). In general, three groups of synthetic fluorogenic and chromogenic substrates can be distinguished (Table II).

(i) Fluorogenic dyes that cause the increase of fluorescence as a result of adsorbance of fluorescent dye onto DNA or protein of bacterial cells.

(ii) pH-Fluorescent indicators. The change in intensity of fluorescence or absorbance of a pH indicator as a consequence of enzymatic activities.

(iii) Enzyme substrates. The substrate complex is hydrolyzed by the appropriate enzyme and free chromogen or fluorogen is released, which can be

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detected either colorimetrically or fluorometrically. Chromogenic enzyme substrates are mainly phenol derivatives, such as o-, and p-nitrophenols, indoxyl, phenolphthalein and p-nitroaniline compounds. Most fluorogenic enzyme substrates are derivated from coumarin, such as 4-methylumbelliferone (4-MU) or 7-amino-4-methylcoumarin (7-AMC) as well as derivatives from beta-naphthylamine (beta-NAP). Several substrates can be incorporated into media or detection kits for rapid and direct identification of microorganisms. This subject has been reviewed in detail recently /1/.

Table I

Application of fluorogenic and chromogenic
substrates in microbiology

Direct detection of microorganisms in media
Enzyme profile investigation
Epifluorescence microscopy
Direct epifluorescence filter technique (DEFT)
Flow cytometry (FCM)
Fluorescent immunoassay (FIA)
Fluorescent microscopy
Fluorometric and photometric methods in microbiology
Automation using chromogenic and fluorogenic substrates

Table II

Fluorogenic and chromogenic substrates

FLUOROGENIC DYES:
8-Anilino-1-naphthalene-sulfonic acid (ANS)
Acridine-orange (AO)
pH-FLUORESCENT INDICATORS: Acridine, 7-Hydroxycoumarin
ENZYME SUBSTRATES: o- or p-Nitrophenol
5-Bromo-4-chloro-3-indolyl-(X)
4-Methylumbelliferone- (4MU)
7-Amido-4-methylcoumarin- (7AMC)

Direct detection of microorganisms in media

Identification of bacteria is usually based on diagnostic schemes using extensive batteries of conventional tests. Common to these methods is that they require a single and pure bacterial colony taken from primary isolation plate. In most cases, diagnosis is therefore time-consuming, inconvenient, and requires several steps of handling.

The ability to detect the presence of a specific and exclusive enzyme using suitable substrates, in particular fluorogenic enzyme substrates, has led to the development of a great number of methods for the identification of microorganisms even in primary isolation media. The incorporation of chromogenic or fluorogenic enzyme substrates into a selective medium can eliminate the need for subculture and further biochemical tests. Based on the visible colour or fluorescence, detectable under long-wave ultraviolet light, it is possible to identify certain microorganisms.

The total coliforms and particularly Escherichia coli are treated as indicator organisms for faecal pollution, therefore the detection and enumeration of these bacteria is of primary importance for microbiological quality control of foods and water. The most relevant test used for the enumeration of the coliform group is the hydrolysis of lactose. The breakdown of this disaccharide is catalyzed by the enzyme beta-D-galactosidase; and its determination is accomplished by using substrates such as o-nitrophenyl-beta-D-galactopyranoside (ONPG), 6-bromo-2-naphthyl-beta-galactopyranoside or 4-methylumbelliferyl-beta-D-galactopyranoside (MUGA) (Table III). The formation of fluorescent 4-methylumbelliferone (4-MU) from nonfluorescent 4-MU-beta-D-galactopyranoside is taken as a measure for beta-D-galactosidase activity.

Other screening methods for the diagnosis of Enterobacteriaceae make use of the detection of caprylate esterase using 4-methylumbelliferyl-cap-

Table III
Application of enzyme tests to members of the
family Enterobacteriaceae

<u>β-D-Galactosidase</u>	Coliforms
5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside	(XGAL)
o-Nitrophenyl- β -D-galactopyranoside	(ONPG)
6-Bromo-2-naphthyl- β -D-galactopyranoside	(BNGA)
4-Methylumbelliferyl- β -D-galactopyranoside	(MUGA)
<u>Phenylalanine deaminase</u> <u>Proteus</u> , <u>Morganella</u> , <u>Providencia</u>	
p-Nitro-DL-phenylalanine	
Test: Pathotec (General Diagnostics)	
<u>Caprylate esterase</u>	<u>Salmonella</u> spp.
4-Methylumbelliferyl-caprylate	
Test: MUCAP Test (Biolife, Italy)	

rylate for Salmonella spp. (MUCAP test), and of phenylalanine deaminase for the detection of Proteus, Morganella and Providencia group.

Beta-D-glucuronidase (GUD) is an E. coli specific enzyme and present in 96-98% of these strains (with exception of E. coli 0157:H7). It can, therefore, be utilized for specific identification of E. coli. GUD activity is measured by using different chromogenic and fluorogenic substrates (Table IV). For detection of E. coli in various specimens, either a yellow coloration of the liquid growth medium due to formation of p-nitrophenol released from p-nitrophenol-beta-D-glucuronide (PNPG) or the production of blue-coloured colonies on the agar plate using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (XGLU) can be applied. As an alternative to these chromogenic procedures, fluorogenic 4-methylumbelliferyl-beta-D-glucuronide (MUG) is used with high sensitivity. This fluorogenic substrate is broken down by GUD to release 4-MU which fluoresces blue under long-wave UV light /1, 2/. Commercial MUG media are available such as Fluorocult^R culture media /3-5/ and as Bactident E. coli test /6/ (Table IV).

Table IV

Application of enzyme tests to specific taxa - "E. coli"

β -D-Glucuronidase Test

p-Nitrophenyl- β -D-glucuronide	(PNPG)
5-Bromo-4-chloro-3-indolyl- β -D-glucuronide	(XGLU)
4-Methylumbelliferyl- β -D-glucuronide	(MUG)

<u>Tests</u>	Fluorocult media & Bactident <u>E. coli</u> (Merck)
	Rapidec coli (bio Merieux)
	MUG Plus Test (Difco Laboratories)
	Lyfokkwik OMI (Micro-Bio-Logic)
	RIM <u>E. coli</u> Swab (Austin Biological Lab.)
	Rapid Detect <u>E. coli</u> Test (Organon Teknika Corp.)
	PNPG disks (Rosco)
	MUG Tube Test (Remel)
	Hach MPN system (Hach)

Several attempts have been made to detect coliforms and E. coli simultaneously. New methods have been introduced that are based on the detection of beta-D-galactosidase and beta-D-glucuronidase using a combination of MUG-ONPG or an improved method using MUG-XGAL (Table V). When using 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (XGAL) directly incorporated into liquid or solid media, a rapid and sensitive detection of coliforms and E. coli was achieved /7/.

Table VThe simultaneous detection of total coliforms and *E. coli*

<u>β-D-Galactosidase</u>	
5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside	(XGAL)
o-Nitrophenyl- β -D-galactopyranoside	(ONPG)
<u>β-D-Glucuronidase</u>	
4-Methylumbelliferyl- β -D-glucuronide	(MUG)
<u>Tests</u> MUG/ONPG (Colilert, Access & Coliquick, Hach)	
MUG/XGAL (EMX-agar plate; Biotest, Austria)	
<u>Reference</u> /7/	

Table VIApplication of fluorogenic and chromogenic substrates to the identification of cocci

<u>α-Galactosidase</u>	<u><i>Bifidiobacterium</i> spp.</u> lactic acid bacteria <u><i>S. bovis</i> and <i>E. faecalis</i></u>
5-Bromo-4-chloro-3-indolyl- α -D-galactopyranoside	
4-Methylumbelliferyl- α -D-galactoside	
<u>Test</u> CPS ID agar (bioMérieux)	

Table VIIIdentification of enterococci and *S. pyogenes* — "PYRase" Test

<u>Pyroglutamyl aminopeptidase</u>	enterococci <u><i>S. pyogenes</i></u>
L-Pyroglutamyl-p-nitroanilide	
L-Pyroglutamyl-7-amido-4-methylcoumarin	
L-Pyrrolidonyl- β -naphthylamide	
<u>Tests</u> Strep-A-Fluor paper strip (Bio Spec USA)	
Identicult-AE, (Scott Lab. Inc., Fiskeville)	
Minitek PYR disks (BBL)	
Strep-A-Chek (EY Lab., CA, USA)	

Enzyme-based tests for the enumeration of cocci are summarized in Tables VI to VIII. For the differentiation of group A streptococci and enterococci the measurement of pyroglutamyl-aminopeptidase (PYRase) was shown to be a useful alternative to conventional methods /8, 9/. Several authors

Table VIIIEnzyme tests for the identification of *S. aureus*DNAse test

DNA agar +
 5-Monophosphate p-nitrophenyl ester ammonium salt
 5-Bromo-4-chloro-3-indolyl-thymidine-3-phosphate
 Toluidine blue or methyl green or acridine orange

Staphylocoagulase

D-Phe-Pro-Arg- β -naphthylamine
 Chromozym TH (N-tosyl-glycyl-L-prolyl-L-arginyl-
 p-nitroanilide.hydrochloride)

Thermonuclease

DNA agar and ethidium bromide

described techniques utilizing L-pyrrolidonyl-beta-naphthylamide (PYR) as a specific substrate for this assay. Other researchers used chromogenic pyroglutamic-acid-p-nitroanilide or fluorogenic L-pyroglutamic-acid-7-amido-4-methyl-coumarin (Table VII).

The examination of DNAse and coagulase activities is essential for the identification of Staphylococcus aureus. Tests commonly applied for the assessment of the DNAse reaction are based on the hydrolysis of natural DNA. Toluidine blue, methyl green, acridine orange and 5-bromo-4-chloro-4-indolyl-thymidine-3-phosphate are used as ingredients of DNA containing agars in order to directly detect DNAse activity. According to Bulanda et al. /10/, the chromogenic substrate D-phe-pro-arg-beta-naphthylamide could be used for the detection of staphylocoagulase of S. aureus (Table VIII).

By using p-nitrophenyl-N-acetyl-beta-D-galactosaminide or 4-methylumbelliferyl-N-acetyl-beta-D-galactosaminide for detection of N-acetyl-beta-D-galactosaminidase rapid identification of Candida albicans is accomplished. 4-MU-N-acetyl-beta-D-galactosaminide is incorporated into a solid medium such as the Fluoroplate^R Candida Agar (Merck), as a reagent or as a paper strip test (Table IX) /11/.

β -L-Glutamyl-arylpeptidase can be used for identification of Neisseria meningitidis, and L-hydroxyproline-arylpeptidase for the examination of Neisseria gonorrhoeae. 4-Methylumbelliferyl-butyrate or indoxyl-butyrate is taken as a specific substrate for rapid identification of Branhamella catarrhalis based on its butyrate esterase activity (Table X).

Table IX

Candida albicans

Fluorogenic dye: Aniline blue dye medium, C. albicans and C. stellatoidea fluoresce yellow-green under UV light (365 nm)

N-Acetyl- β -D-galactosaminidase

4-Methylumbelliferyl-N-acetyl- β -D-galactosaminide
p-Nitrophenyl-N-acetyl- β -D-galactosaminide

Tests Fluoroplate Candida Agar (Merck, Germany)
MJAG Candi Kit (Biolife, Italy)
Alpistrip (Lab. M. Ltd., UK)

Reference /11/

Table X

Tests for differentiating between Neisseria
and related species

<u>Butyrate esterase</u>	<u>Branhamella catarrhalis</u>
4-Methylumbelliferyl butyrate	
Indoxyl butyrate	
<u>Test</u> Butylase strip (Lab M., UK)	
<u>β-D-Galactosidase</u>	<u>N. lactamica</u>
<u>L-L-Glutamylaminopeptidase</u>	<u>N. meningitidis</u>
<u>L-Hydroxypropylaminopeptidase</u>	<u>N. gonorrhoeae</u>
<u>Tests</u> Gonocheck II (Du Pont)	
Identicult Neisseria (Scott Lab.)	
Rapid N/H (Innovative Diag.)	
HNID Panel (Micro Scan, Baxter)	
NH ID card (Vitek)	

The combination of different enzymes and substrates for simultaneous detection of bacteria in the same medium has been applied successfully for the detection of urinary tract infections such as E. coli, Proteus and enterococci. Different enzymes such as beta-D-glucuronidase, beta-D-galactosidase, tryptophan-deaminase, tryptophanase and esculinase are used in these tests (Table XI).

Further applications of enzyme substrates are made to distinguish Gram-positive from Gram-negative bacteria, based on L-alanine-aminopeptidase activity of the Gram-negative bacteria. We have found that the fluorogenic L-alanine-7-amido-4-methylcoumarin (AAMC) is more sensitive than other en-

Table XI
Detection of urinary tract infections by
rapid enzymatic methods

CPS ID agar & Uriline (Api, bioMérieux, France)
Agar dip-slide (Diagnostics Pasteur)
Chemstrip LN (Bio-Dynamic, Ind., USA)
Rapid Urine System (Miro-Bio-Logic, Minn. USA)
Urotube Coli and Urotube Enterococci (Hoffmann-LaRoche)

zyme substrates /12/. Incorporating AAMC into growth media, Gram-negative bacteria give blue fluorescent colonies on plates or bluish fluorescence in broth when illuminated with long-wave UV light. Rapid Gram differentiation is also enabled by using commercially available test strips impregnated with the chromogenic L-alanine-4-nitroanilide (Bactident^R Aminopeptidase, Merck). Another substrate used for Gram differentiation is the fluorogenic protein-specific dye 8-anilino-1-naphthalene sulphonic acid (ANS) (Table XII).

Table XII
Gram differentiation of bacteria

<u>L-Alanine-aminopeptidase</u>	
L-Alanine-4-nitroanilide	(LANA)
L-Alanine-7-amido-4-methylcoumarin	(AAMC)
L-Alanine-4-methoxy- β -naphthylamide	(MNA)
4-Alanine-2-amidoacridone	(AAA)
<u>Test Bactident Aminopeptidase (Merck)</u>	
<u>Fluorogenic dye</u>	
8-Anilino-1-naphthalene-sulphonic acid	(ANS)
<u>Reference /12/</u>	

Table XIII
Other enzyme tests

<u>Chitinase activity</u>
4-methylumbelliferyl-N-acetyl- β -D-glucosaminide
<u>Sialidase (neuraminidase) activity</u>
2-(4-methylumbelliferyl) alpha-D-N-acetylneuraminidase
<u>Trypsin-like activity in <i>Vibrio parahaemolyticus</i></u>
benzyl-L-arginine-7-aminomethyl coumarin

Other enzymes such as glycosidases, arylamidases, esterases or phosphatases found in other bacteria can be detected by use of specific fluorogenic substrates (Table XIII).

Fluorometric and photometric methods in microbiology

Fluorometric and colorimetric methods detecting certain enzymatic activities such as beta-D-glucuronidase, beta-D-galactosidase, or pyroglutamyl-aminopeptidase in samples without culturing, may be considered as an additional approach in modern microbiological examination. At present, these methods are in stage of development and can be used particularly in food and water microbiology, e.g. for detecting E. coli /13/, coliforms /14/ and Enterococcus spp. /8/.

Enzyme profiles as an aid in microbiological taxonomy

In general, microorganisms are classified on the basis of overall morphological and biochemical characteristics; definitive taxonomic assignment is determined by nucleic acid hybridization. This technique is laborious and time-consuming and, therefore, not amenable to routine analysis. Alternatively, definition of enzyme profiles by applying electrophoretic separation or enzyme substrate kits, which gives results in good agreement with genetic analysis, has been used in several studies. The characterization of bacteria based on the enzyme patterns using, for instance, cluster analysis offers new possibilities in microbiological diagnosis. Commercially available kits containing chromogenic enzyme substrates have considerably simplified the use of enzymatic tests and have been frequently used in studies on microbiological taxonomy.

Automation

In the clinical laboratory, the improved identification of microorganisms has been facilitated by the availability of rapid and miniaturized kit identification systems, which may be processed and interpreted by automated instrumentation combined with a data management system. Some automated microbiology systems such as the Vitek AutoMicrobic System (Vitek AMS; Hazelwood, MO, USA), the API ALADIN (Analytab Products; Plainview, N.Y., USA), and the Auto SCAN-W/A System (MicroScan Division, Baxter Diagnostics, CA,

USA) have been introduced. The Auto SCAN-W/A system /15/ consists of an automated incubator-interpretation module in combination with a data management system. In the identification panels, a series of chromogenic or fluorogenic substrates are used. The inoculated panels are inserted into the incubation-interpretation module, reagents are dispensed automatically after incubation and the results are given on a display. By using this technique, the detection of microorganisms is enabled within 2 (fluorometric assay) to 4 h (colorimetric assay).

Direct epifluorescent filter technique (DEFT)

Basically, this technique is performed to enumerate fluorescent particles and is used in combination with varying degrees of image analysis. This methodological principle is based on the reaction of the fluorochrome acridine orange (AO) as a nucleic dye with microbial cells concentrated on a membrane filter. The cells are counted by using epifluorescence microscopy. In the DEFT technique, samples are first treated chemically in order to disperse the cells and other aggregates and to increase the efficiency of filtration. Then microbial contents of the sample are concentrated by filtration onto polycarbonate membranes before staining. DEFT enables a differential staining where orange or orange/red fluorescing bacteria are thought to be viable and green fluorescing cells non-viable. DEFT methods have been developed for the microbial examination of several foods. For example, an automated method for enumerating bacteria in milk was described /16/

Flow cytometry (FCM)

FCM is an emerging technology with special emphasis on rapid microbiological testing. It is used for medical applications, but has also been applied for rapid counting of bacteria in pure cultures and environmental, food and industrial specimens. This subject has been reviewed previously /17/. FCM techniques utilizing fluorogenic dyes such as acridine orange (AO), ethidium bromide, DAPI, mithramycin and propidium iodide. In FCM, particles are measured while passing through a highly focused light beam. The use of fluorogenic reagents enables measurement of different specific cell constituents such as proteins, DNA, RNA, surface antigens and specific nucleotide sequences. The capability of simultaneously measuring a number of cell constituents as well as determining the particle size makes this system

an exciting and interesting microbiological tool. Because of the expensive equipment, the application of the FCM technique to bacterial studies has not progressed as much as in other biological fields. FCM techniques have been used for the detection and enumeration of certain bacterial taxa including E. coli, Legionella pneumoniae, and other Legionella spp., Nitrosomonas and Streptococcus pyogenes.

Fluorescent immunoassay (FIA)

Fluorochromes, such as fluorescein isothiocyanate are used for labelling immunoglobulins. The fluorochrome absorbs short-wavelength light and then emits light at a higher wavelength, and hence can be detected by using fluorescent microscopy. Fluorochromes can be coupled to an antibody that binds directly with a target antigen on the cell or to a second antibody that recognizes an antibody produced against the microorganism. Fluorescent immunoassays can be applied to almost any microorganisms where agglutinating antisera are available. A variety of bacterial pathogens such as salmonellae or clostridia can be detected in food and water using FIA methods.

In the microbiological laboratory, specificity, sensitivity, and rapidity represent the most important parameters for microbiological identification. Examples described above emphasize the great potential of different applications of various chromo- and fluorophores in microbiological diagnostics. The use of chromogenic and fluorogenic enzyme substrates and its application in analytical microbiology is in the process of developing. There is no doubt that this technique will become even more sensitive and more accurate than it is at present.

REFERENCES

1. Manafi, M., Kneifel, W., Bascomb, S.: Microbiol Rev 55 (1991).
2. Hartman, P. A.: 5th International Symposium on Rapid Methods and Automation in Microbiology and Immunology. Florenz 1987.
3. Hahn, G.: Milchwissenschaft 42, 434 (1987).
4. Havemeister, G., Aleksic, S., Bockemühl, J., Heinemeyer, E. A., Müller, H. E., von Pritzbuer, E.: Zentralbl Hyg 191, 523 (1991).
5. Heizmann, W., Döller, P. C., Gutbrod, B., Werner, H.: J Clin Microbiol 26, 2682 (1988).
6. Hofmann, O., Rager, K. T.: Wasser und Abwasser 129, 19 (1988).
7. Manafi, M., Kneifel, W.: Zentralbl Hyg 189, 225 (1989).
8. Ellner, P. D., Williams, D. A., Hosmer, M. E., Cohenford, M. A.: J Clin Microbiol 22, 880 (1985).
9. Godsey, J. H., Schulman, R., Eriquez, L.: 81st Annual Meeting of the American Society for Microbiology 1981.

10. Bulanda, M., Wegrzynowicz, Z., Ciurak, M., Kowalczyk, J., Kupryszewski, G., Gruszka Heczko, M. P. B., Pulverer, G.: *Zentralbl Bakteri* 270, 115 (1988).
11. Manafi, M., Willinger, B.: *J. Microbiol Methods*. In Press (1991).
12. Manafi, M., Kneifel, W.: *J Appl Bacteriol* 69, 822 (1990).
13. Adams, M. R., Grubb, S. M., Hamer, A., Clifford, M. N.: *Appl Environ Microbiol* 56, 2021 (1990).
14. Cenci, G., Caldini, G., Sfodera, F., Morozzi, G.: *Microbiologica* 13, 121 (1990).
15. Baxter MicroScan^R, Rapid Identification Procedural Manual. Baxter Diagnostics Inc. West Sacramento CA, USA 1990.
16. Pettipher, G. L.: The direct epifluorescent filter technique for the rapid enumeration of micro-organisms. John Wiley & Sons, Inc., New York 1983.
17. Melamed, M. R., Lindmo, T., Mendelsohn, M. L. (eds): *Flow Cytometry and sorting*. 2nd ed. Wiley-Liss, New York 1990.

RAPID METHODS AND COMPUTER ASSISTED DIAGNOSIS IN MEDICAL MICROBIOLOGY

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(Received September 24, 1991)

Rapid diagnosis and reporting in medical microbiology is becoming more and more important. In recent years, introduction of automated instruments as well as of computer assisted diagnosis contributed to this aim. These methods, however, are very expensive. A more cost efficient and simple to perform method for rapid diagnosis is the use of specific fluorogenic substrates incorporated into culture media (solid or liquid) for identification of the most important pathogens, e.g. Escherichia coli. Investigation of Fluorocult ECD agar and Columbia agar revealed a high sensitivity (85%) and an excellent specificity (> 99%) of fluorescence in combination with a positive indole reaction for identification of E. coli.

Clinical impact of rapid methods

At least in Europe, North America and other industrialized countries (in contrast to developing ones), classical infectious diseases are becoming less frequent. Contrary to that there is an increasing number of immunocompromised patients, including those with organ transplantations, bone marrow transplantations, haematological malignancies with aggressive chemotherapy and also patients with HIV-infections suffering from unspecific infectious diseases. There are a great number of different pathogens, however, mainly part of the endogenous flora of healthy persons. In this patient population it is evident that rapid reporting of microbiological results is one of the most crucial points in acceptance of medical microbiology, especially in improvement of patient care /1/.

Calculated chemotherapy and new β -lactamases

To contribute to this aim, there are several tools. The first step is to create a protocol for a so-called calculated chemotherapy in cooperation

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with clinicians (Fig. 1). Calculated chemotherapy /2, 3/ is intended to improve antimicrobial therapy between onset of disease and the time of reporting of microbiological results. There are at least four components of this approach. The first one is to analyze data of controlled clinical trials already published as well as of microbiological data presenting minimal inhibitory concentrations of a broad range of pathogens including difficult to treat species. Then we should include pharmacological and also pharmacokinetic data. The third component are individual epidemiological data of the hospital, wards (e.g. ICU) or even of a single patient (surveillance cultures /4, 5/). And last but not least, economical considerations are becoming more and more important.

However, the emergence of new types of β -lactamases makes this approach more and more difficult. Common β -lactamases are widely distributed, mostly belonging to plasmid encoded β -lactamases, belonging to class III according to the criteria published by Richmond and Sikes /6/. Fortunately a great number of β -lactam antibiotics is still efficient in these bacteria. In 1983, a new type of β -lactamase was described /7/, today known as "extended broad spectrum β -lactamase" (EBS- β -lactamase), able to inactivate cephalosporins of the cefotaxime type. These EBS- β -lactamases are inhibited by substances like sulbactam and others /8/. Another new type of β -lactamase was described in 1990, similar to class I β -lactamases, but plasmid mediated /9/. Unfortunately, only carbapenems were not affected by this type. Up to now, EBS- and MIR-1- β -lactamases are mainly restricted to a limited number

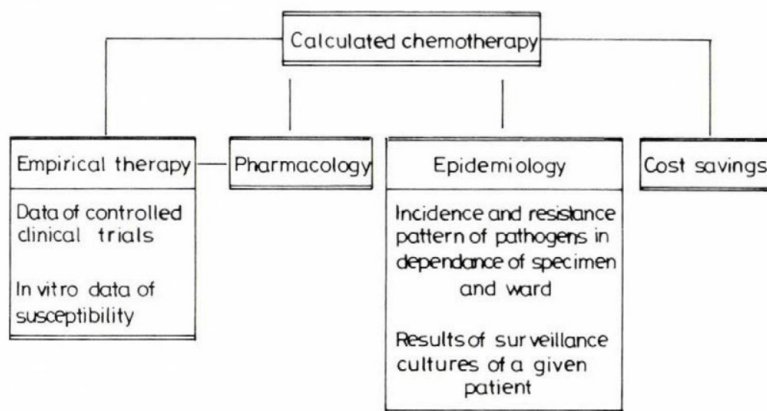


Fig. 1. Components of calculated chemotherapy

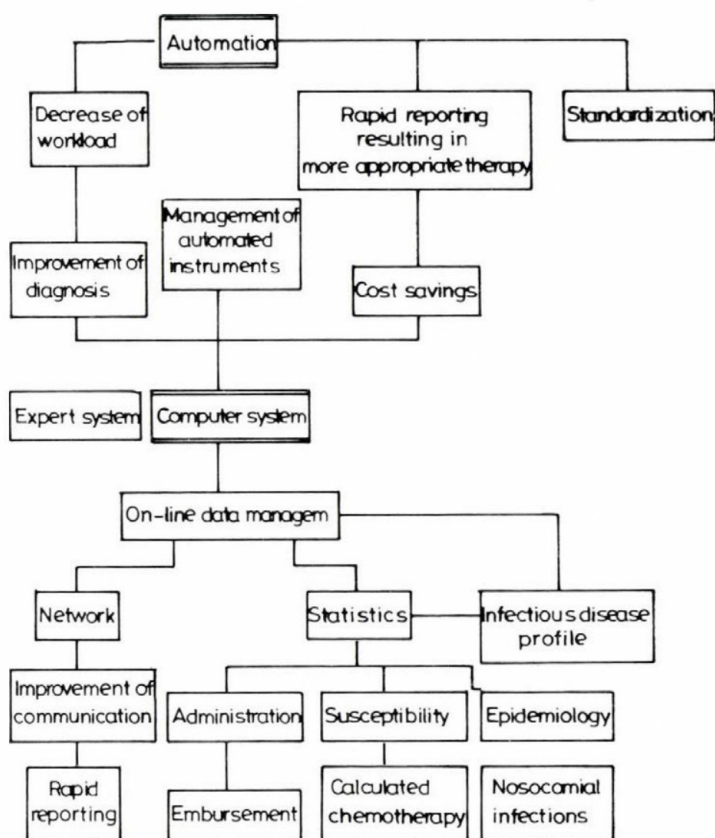


Fig. 2. Aspects of automation and computer assisted diagnosis in medical microbiology

of species within the family *Enterobacteriaceae*, mainly *Klebsiella pneumoniae*. The emergence of such types of resistance, however, adds another argument to know as fast as possible, which pathogen is responsible for the infectious process.

Automated instruments and computer assisted diagnosis

In the past few years, many technological developments like automated instruments as well as application of computer assisted diagnosis [10] made it possible to become faster and faster in medical microbiology (Fig. 2). Automation may lead to a decrease of work-load in the laboratory, thus more sophisticated methods can be implemented (e.g. detection of virulence markers

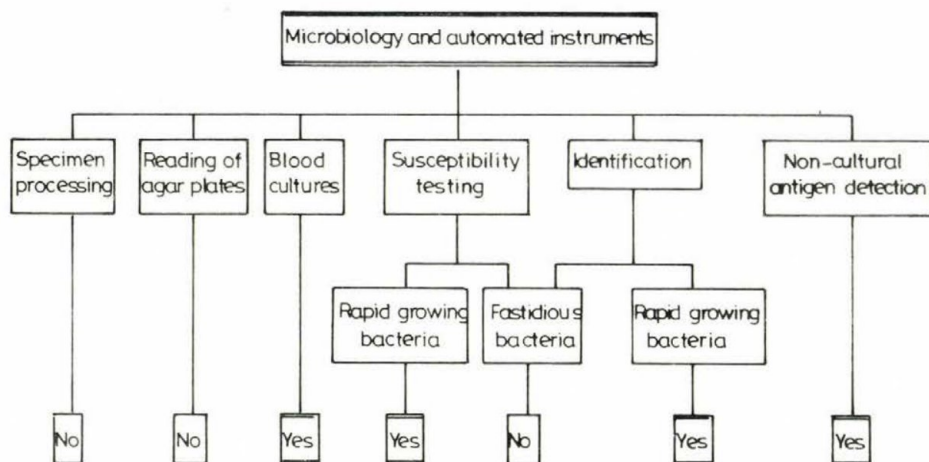


Fig. 3. Possible use of automated instruments

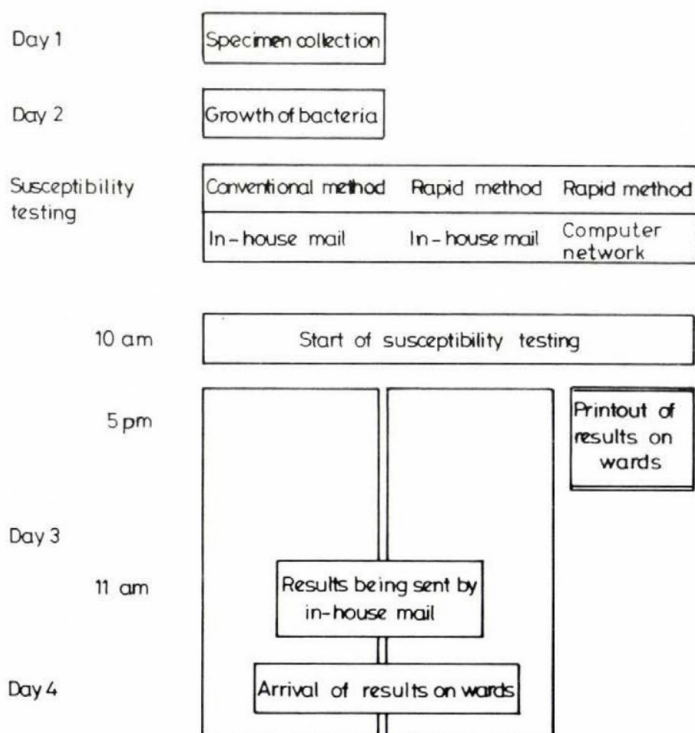


Fig. 4. Time table of reporting microbiological results using different methods

or detection of toxins) resulting in improvement of diagnosis. Many automated instruments are generating data within a few hours, and rapid reporting becomes possible, presumptive resulting in a more appropriate therapy. Today, automated instruments can be used for identification and antimicrobial susceptibility testing of non-fastidious bacteria /11-14/ and in non-cultural antigen detection systems /15/ (Fig. 3). Combination of automated instruments with computer assisted diagnosis may further lead to a much better cooperation with the clinicians. One can generate data for an "infectious disease profile" of immunocompromised patients for the estimation of the individual risk /2/, and also epidemiological data for calculated chemotherapy. If there is a computer network installed within the hospital, significant acceleration of data management is possible (Fig. 4). In general, up to 3 and more days will elapse from sampling of specimen till arrival of microbiological reports on the ward, especially in busy laboratories it is not possible to report all results by phone. Using automated instruments in combination with computer assisted diagnosis, in many cases this times decreases down to just 24 h.

Alternative ways for rapid reporting

This approach, however, is hard to realize, because costs are very high, and one needs at least two years to install these systems within a given hospital. Therefore it seems reasonable to develop methods to improve and accelerate diagnosis. These methods have to fit into laboratory work and costs should be moderate.

Analysis of the most important and also most frequent pathogens in different specimens reveals an almost uniform pattern. If we look at the top ten of the pathogens isolated from various samples excluding blood cultures and urines of an university hospital, we will find Escherichia coli as the leading Gram-negative pathogen, only outnumbered by Staphylococcus aureus and coagulase negative staphylococci (Table I). The same pattern as before can be found in blood cultures /16, 17/. It has also been long known that E. coli is the most important cause of urinary tract infections (Table II). This holds true for hospitalized as well as out-patients.

Thus, rapid identification of E. coli, the leading Gram-negative pathogen by simple and easy to perform methods can improve diagnosis significantly. One of these methods is the use of media with incorporation of a substrate like 4-methylumbelliferyl- β -D-glucuronide (MUG; Fluorocult media), cleaved

Table I

Top ten of pathogens,
University Hospital Tübingen

Without urines and blood cultures; total number of strains, 16380

<u>S. aureus</u>	3701
<u>S. epidermidis</u>	2281
<u>E. coli</u>	2080
<u>P. aeruginosa</u>	1234
<u>E. faecalis</u>	1043
<u>K. oxytoca</u>	745
<u>S. agalactiae</u>	600
<u>K. pneumoniae</u>	565
<u>E. cloacae</u>	529
<u>P. mirabilis</u>	468

Table II

Top ten of pathogens,
University Hospital Tübingen

Urine specimens, total number of strains, 5380

<u>E. coli</u>	2241
<u>E. faecalis</u>	969
CNA	473
<u>S. pyogenes</u>	247
<u>P. aeruginosa</u>	239
<u>S. aureus</u>	200
<u>K. pneumoniae</u>	195
<u>Citrobacter</u> spp.	53
<u>E. cloacae</u>	52
<u>K. oxytoca</u>	46

by an enzyme (glucuronidase) of E. coli to produce fluorescent 4-methylumbelliferon.

Table III

Enterobacteriaceae — investigation of Fluorocult
ECD-agar for identification of E. coli

	Gold standard		Total
	<u>E. coli</u>	Not <u>E. coli</u>	
MUG+/Indole+	254	2	256
MUG-	42	540	582
Total	296	542	838
Sensitivity	85%	Specificity	>99%

Table IV
Enterobacteriaceae — investigation of Fluorocult
Columbia Agar with 5% sheep blood for identification
of *E. coli*

	Gold standard		Total
	<u><i>E. coli</i></u>	Not <u><i>E. coli</i></u>	
MUG+/Indole+	253	2	255
MUG-	43	540	583
Total	296	542	838
Sensitivity	85%	Specificity	> 99%

In a small investigation (with a total of 1258 Gram-positive and -negative strains), we studied the performance of the Fluorocult ECD-agar as well as of the Fluorocult Columbia Agar. The data we found for ECD were quite excellent in comparison to the gold standard, that is identification

Table V
Gram-negative rods other than Enterobacteriaceae —
performance of Fluorocult media

	No. of strains	MUG positive	
<u><i>P. aeruginosa</i></u>	23	9,	but definitively distinct
<u><i>P. maltophilia</i></u>	21	0	
<u><i>P. paucimobilis</i></u>	1	0	
<u><i>P. testosteronis</i></u>	1	0	
<u><i>P. luteola</i></u>	1	0	
<u><i>P. pseudoalcaligenes</i></u>	9	0	
<u><i>P. stutzeri</i></u>	8	0	
<u><i>P. oryzae</i></u>	4	0	
<u><i>P. putrefaciens</i></u>	1	0	
<u><i>P. putida</i></u>	7	0	
<u><i>P. diminuta</i></u>	3	0	
<u><i>P. fluorescens</i></u>	7	0	
<u><i>P. acidovorans</i></u>	2	0	
<u><i>P. cepacia</i></u>	2	0	
<u><i>Acinetobacter</i> spp.</u>	77	1,	but indole-negative
<u><i>Pasteurella</i> spp.</u>	3	0	
<u><i>A. hydrophila</i></u>	6	0	
<u><i>F. meningosepticum</i></u>	12	0	
<u><i>Alcaligenes</i> sp.</u>	2	0	
<u><i>Moraxella</i> spp.</u>	6	0	
<u><i>B. fragilis</i></u>	20	0	
<u><i>B. thetaiotaomicron</i></u>	20	0	

according to the methods given by Farmer et al. /18/. The sensitivity was 85%, with a specificity of greater 99% (Table III), even better than in identification systems like api /14/. False positive identifications were found in one strain each of Morganella morganii and Serratia marcescens.

Identical figures as for ECD agar revealed investigation of Columbia Agar (Table IV), with a sensitivity of 95% and a specificity of greater 99%. False positive results were found in one strain each of Klebsiella oxytoca and S. marcescens. Besides Enterobacteriaceae we also investigated a great number of other Gram-negative rods including anaerobes, with no false positive results of the combination MUG-positive, indole-positive (Table V).

Fluorescent colonies were detected in some positive cocci, but this will have no impact on diagnosis, because colony morphology is quite distinct from E. coli. In the case of coagulase-negative staphylococci one even can use a positive fluorescence for differentiation.

In conclusion, Fluorocult media have a very good sensitivity and excellent specificity for identification of the leading Gram-negative pathogen, E. coli, and one can rule out other species of Enterobacteriaceae in the shortest time possible. Using this simple and easy to perform method of cultivation makes it also possible, to accelerate diagnosis, thus improving cooperation between microbiologist and clinician for the sake of a better patient care.

REFERENCES

1. Trenholme, G. M., Kaplan, R. L., Karakusis, P. H., Stine, T., Fuhrer, J., Landau, W., Levin, S.: J Clin Microbiol 27, 1342 (1989).
2. Heizmann, W. R.: Internist 31, 438 (1990).
3. Sturm, A. W.: Eur J Clin Microbiol Infect Dis 9, 381 (1990).
4. Heizmann, W. R., Zabel, L., Krüger, H.-U.: In Werner, H., Heizmann, W. R. (eds): Infektiologische Probleme bei Patienten auf Intensivstationen. Schattauer, Stuttgart-New York 1979. pp. 65-73.
5. Daw, M. A., Munnelly, P., McCann, S. R., Daly, P. A., Falkiner, F. R., Keane, C. T.: Eur J Clin Microbiol Infect Dis 7, 742 (1988).
6. Richmond, M. H., Sykes, R. B.: In Rose, A. H., Tempest, D. W. (eds): The β -lactamases of Gram-negative bacteria and their possible physiological role. Academic Press, London-New York 1973. pp. 31-88.
7. Knothe, H., Shah, P., Krcmery, V., Antal, M., Mitsuhashi, S.: Infection 11, 35 (1983).
8. Legrand, P., Fournier, G., Bure, A., Jarlier, V., Nicolas, M. H., Decre, D., Duval, J., Philippon, A.: Eur J Clin Microbiol Infect Dis 8, 527 (1989).
9. Papanicolaou, G. A., Medeiros, A. A., Jacoby, G. A.: Antimicrob Agents Chemother 34, 2200 (1990).
10. Heizmann, W., Pickert, A., Klöss, Th., Werner, H.: Infection 16, 69 (1988).
11. Wüst, J., Heizmann, W., Hardegger, U., Manncke, B.: Eur J Clin Microbiol Infect Dis 7, 511 (1988).

12. Szeto, S., Luie, M., Low, D. E., Patel, M., Simor, A. E.: J Clin Microbiol 29, 1258 (1991).
13. Truant, A. L., Starr, E., Nevel, C. A., Tsolakis, M., Fiss, E. F.: Diagn Microbiol Infect Dis 12, 211 (1989).
14. Heizmann, W. R.: Computergestützte Diagnosesysteme und Automation. Wissenschaftliche Verlagsgesellschaft, Stuttgart 1989.
15. Ortisis, G., Nicastro, G., Rigoli, R., Matinato, C., Scarpellini, P., Magliano, E. M., Privitera, G.: Abstr. 91st Gen Meet Am Soc Microbiol 1991 C-100, 358 (1991) (Abstract).
16. Heizmann, W., Ostendorf, P., Klöss, Th., Botzenhart, K.: Lab Med 2, 276 (1985).
17. Murray, P. R., Spizzo, A. W., Niles, A.: J Clin Microbiol 29, 901 (1991).
18. Farmer, J. J. III, Davis, B. R., Hickmann-Brenner, F. W. et al.: J Clin Microbiol 21, 46 (1985).

PATHOGENIC ESCHERICHIA COLI 0157:H7 AND THEIR DETECTION

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(Received September 24, 1991)

Enterohaemorrhagic Escherichia coli 0157:H7 is of major concern to the food industry due to high pathogenicity of this foodborne organism. For the detection of these bacteria a special agar medium with a fluorogenic substrate has been developed. The medium uses the characteristics of this E. coli serotype not to ferment sorbitol and not to produce β -glucuronidase. In contrast, approximately 96% of all other strains of E. coli are sorbitol-positive and nearly all of them are β -glucuronidase-positive. For discrimination between Proteus and E. coli 0157:H7 which are both sorbitol- and β -glucuronidase-negative, sodium thiosulphate and ferric ammonium citrate were added. This leads to a brownish colour of the Proteus colonies due to their production of hydrogen sulphide. Growth of the gram-positive flora was inhibited by the addition of sodium deoxycholate.

Escherichia coli 0157:H7 has been shown to be the causative agent of several severe foodborne outbreaks of haemorrhagic colitis and haemolytic uraemic syndrome leading to death in many cases. These bacteria were first isolated from outbreaks in Oregon and Michigan, where contaminated hamburgers seemed to be the spreaders of the agent /1/. Other outbreaks have been traced back to the consumption of contaminated ground beef /2-4/. But also raw milk and vegetables have been implicated as vehicles of E. Coli 0157:H7 /5, 6/. Several studies in the last few years presented evidence that the main reservoirs of enterohaemorrhagic E. coli are domestic animals, mainly cattle /7-9/. Thus, investigation of food of animal origin for this serotype of E. coli becomes more and more necessary in the microbiological quality control of foods.

A common method to isolate this type of E. coli is based on the characteristic of these strains not to ferment sorbitol /10-13/. Whereas 96% of

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apathogenic E. coli are sorbitol-positive all known strains of E. coli O157:H7 are sorbitol-negative. However, this method is still very time consuming and many false positive results are obtained due to the relative high incidence of sorbitol-negative apathogenic E. coli and other sorbitol-negative Enterobacteriaceae.

In this publication an improved agar medium for the detection of E. coli O157:H7 is described. The medium is selective for Gram-negative bacteria and contains in addition to sorbitol the fluorogenic indicator substance 4-methylumbelliferyl- β -D-glucuronide (MUG). This molecule is cleaved by the enzyme β -D-glucuronidase to the fluorescent compound 4-methylumbelliferone. Nearly all strains of E. coli express this enzyme. In contrast, none of the described O157:H7 strains has been shown to be β -D-glucuronidase-positive /14, 15/. Furthermore, the medium was supplemented with indicators for hydrogen sulphide production for differentiation between Proteus and E. coli O157:H7. The indicator dye bromthymol blue was added to demonstrate sorbitol fermentation and acidification of the colonies by colour change from green to yellow.

Materials and methods

Bacteria. E. coli O157:H7 strains were kindly provided by Dr. Karch, Institute of Hygiene and Microbiology, University of Würzburg, Germany. All other strains were from the ATCC or the DSM.

Media. Media used as well as chemicals and the UV-lamp were from E. Merck, Darmstadt, Germany.

Composition of Fluorocult E. coli O157:H7. Based on the formula of Szabó et al. /15/ a medium was developed. The medium contains g/litre: peptone from caseine, 20.0; meat extract, 2.0; yeast extract, 1.0; sorbitol, 10.0; ferric ammonium citrate, 0.5; sodium thiosulphate, 2.0; 4-methylumbelliferyl- β -D-glucuronide, 0.1; sodium chloride, 5.0; sodium desoxycholate, 1.12; bromthymol blue, 0.025; agar, 13.0; pH 7.3–7.5; 121 °C 15 min. Distributed in Petri dishes.

Preparation of meat samples. E. coli O157:H7 were grown overnight in CASO Broth at 37 °C. Cultures were serially diluted in 0.9% NaCl solution and 0.1 ml aliquots were inoculated into retail ground beef that was formed to balls of 25 g. Inoculated balls were frozen for 3 days.

Recovery of E. coli O157:H7 from beef. Beef samples were transferred into bags containing 225 ml Lauryl Sulfate Broth. The samples were stomached in a Stomacher Lab-Blender 400 for 1 min and incubated at 37 °C for 16–24 h. Before and after enrichment 0.1 ml of each culture was serially diluted in 0.9% NaCl and plated on the new Fluorocult E. coli O157:H7 Agar. Agar plates were incubated at 37 °C for 16–24 h.

Results

Growth of Enterobacteriaceae on Fluorocult E. coli O157:H7 Agar.
Growth of different members of the genus Enterobacteriaceae is summarized in

Table I
Growth of Enterobacteriaceae on Fluorocult E. coli 0157:H7 Agar

Strain	Colonies
<u>E. coli</u> 0157:H7	green, MUG negative
Sorbitol-positive <u>E. coli</u>	yellow, MUG positive
Sorbitol-negative <u>E. coli</u>	green, MUG positive
<u>Enterobacter</u>	yellow, MUG negative
<u>Klebsiella</u>	yellow, MUG negative
<u>Citrobacter</u>	yellow w/ black center, MUG negative
<u>Salmonella</u>	black, MUG positive or negative
<u>Proteus</u>	brown, MUG negative
<u>S. sonnei</u>	green, MUG positive
<u>S. flexneri</u>	no growth

Table I. All E. coli 0157:H7 strains grew as green colonies and did not show any fluorescence. All other strains of E. coli grew as yellow or green colonies and demonstrated strong fluorescence. Enterobacter and Klebsiella colonies were of yellow colour but without fluorescence. Citrobacter and Salmonella colonies were yellow with a black center. After prolonged incubation these colonies were totally black. In addition, some strains of Salmonella exhibited fluorescence. Proteus, which is also a sorbitol-negative organism, grew as brownish colonies. In contrast to Salmonella and Citrobacter, the brownish colour diffused into the surrounding of the colonies. Pseudomonas and Shigella (with the exception of Shigella sonnei) did not ferment sorbitol and were of green colour with no fluorescence. S. sonnei colonies were green but with a bright fluorescence.

Comparison of MacConkey Sorbitol Agar and Fluorocult E. coli 0157:H7 Agar. Growth of different Enterobacteriaceae on MacConkey Sorbitol Agar and the new Fluorocult Agar was compared with respect to differentiation of the species. On MacConkey Sorbitol Agar no difference could be seen between E. coli 0157:H7 and Proteus mirabilis as both species grew as colourless colonies on this medium. On the Fluorocult medium these strains could be distinguished due to hydrogen sulphide production and brownish colour of P. mirabilis. E. coli 0157:H7 and S. sonnei grew both as colourless colonies on MacConkey Sorbitol Agar and as green colonies on Fluorocult medium but could be differentiated by MUG reaction of S. sonnei. The same reaction could be demonstrated for sorbitol-negative but MUG-positive non-0157:H7 E. coli strains. Sorbitol-positive E. coli and Salmonella grew as dark red colonies on MacConkey Sorbitol. On the Fluorocult medium these strains could be distinguished on the basis hydrogen sulphide production by Salmonella.

Table II

Isolation of E. coli O157:H7 from ground beef

Isolation from fresh beef	Total c.f.u./ml t_0	c.f.u. <u>E. coli</u> O157:H7	Recovery of O157:H7
Ground beef uninoculated	9.0×10^3	-	0%
Ground beef + 6.2×10^4 cells per bag		6.8×10^4 cells per bag	109.7%
Ground beef + 6.2×10^5 cells per bag		6.5×10^5 cells per bag	104.8%

Isolation from frozen beef	Total c.f.u./ml t_0	c.f.u./ml total $t_{24\text{ h}}$	c.f.u./ml <u>E. coli</u> O157:H7 $t_{24\text{ h}}$
Ground beef uninoculated	1.1×10^4	7.4×10^8	0
Ground beef + 8 cells		6.8×10^8	2.0×10^7
Ground beef + 61 cells		6.7×10^8	8.0×10^7

Isolation of E. coli O157:H7 from beef. Beef samples were artificially contaminated with different numbers of E. coli O157:H7. Samples were then inoculated on Fluorocult Agar. In frozen beef samples that contained only very low numbers of E. coli O157:H7 (8 and 61 cells per 25 g) enterohaemorrhagic E. coli could be detected on Fluorocult Agar after enrichment of the samples in LSB. From fresh beef that contained 6.5×10^4 or 6.5×10^5 E. coli O157:H7 per 25 g detection of enterohaemorrhagic E. coli was possible without sample enrichment. Recovery was approximately 100% (Table II).

Discussion

The foodborne pathogen E. coli O157:H7 evolves more and more as a serious health problem and the incidence of enteric infections due to this organism ranges now on the third place just below Campylobacter ssp. and Salmonella ssp. induced infections /16/.

A common method to detect E. coli O157:H7 is based on the characteristic of these strains not to ferment sorbitol whereas nearly all other E. coli

serotypes are sorbitol-positive. MacConkey Sorbitol Agar has evolved as the method of choice for the isolation of enterohaemorrhagic E. coli /17/. Sorbitol-negative presumptive E. coli 0157:H7 colonies have to be isolated from this agar and confirmed serologically with 0157 and H7 specific antisera. However, this method is still very time consuming, laborious and expensive. Due to the relative high incidence of sorbitol-negative apathogenic E. coli and other Enterobacteriaceae many colonies will be agglutinated that fail to be 0157:H7.

To reduce the number of false positive results and to make the detection of E. coli 0157:H7 easier, we have developed a medium that contains in addition to sorbitol the fluorogenic substance 4-methylumbelliferyl- β -D-glucuronide (MUG). The nonfluorescent MUG is converted to a fluorescent compound by the enzyme β -D-glucuronidase. This enzyme is produced by nearly all E. coli with the exception of E. coli 0157:H7. Thus, only colonies that are sorbitol and MUG negative are presumptive 0157:H7 strains. A similar agar medium has been described by Szabó et al. /15/ and Okrend et al. /14/. In contrast to Szabó, Okrend et al. used the chromogenic compound BCIG instead of MUG. Okrend et al. demonstrated that the use of an indicator for the enzyme β -D-glucuronidase significantly reduced the number of false positive results.

For further improvement of the medium we have added an indicator for hydrogensulphide production. With this method sorbitol- and MUG-negative Proteus colonies can be easily distinguished from E. coli 0157:H7. Furthermore, addition of this indicator helps to identify Salmonella that can not be differentiated from sorbitol-positive Enterobacteriaceae on MacConkey Sorbitol agar.

With the new Fluorocult Agar medium we were able to detect E. coli 0157:H7 in contaminated beef samples. Enrichment of the bacteria in LSB was necessary only when the contamination rate was very low.

These results clearly demonstrate that the new Fluorocult medium is very efficient in the detection of enterohaemorrhagic E. coli 0157:H7.

Acknowledgement. I thank MICHAEL GAMPE for excellent technical support.

REFERENCES

1. Riley, L. W., Remis, R. S., Helgerson, S. D. et al.: N Engl J Med 308, 681 (1983).
2. Hocking, J. C., Lior, H.: Can Dis Weekly Rep 13, 203 (1987).
3. Ostroff, S. M., Griffin, P. M., Tauxe, R. V. et al.: Am J Epidemiol 132, 239 (1990).

4. Taylor, C. M., White, R. H., Winterborn, M. H. et al.: *Br Med J* 292, 1513 (1986).
5. Duncan, L., Mai, V., Carter, J. et al.: *Can Dis Weekly Rep* 13, 5 (1987).
6. Morgan, G. M., Newman, C., Palmer, S. R. et al.: *Epidemiol Infect* 101, 83 (1988).
7. Borczyk, A. A., Karmali, M. A., Lior, H. et al.: *Lancet* 1, 98 (1987).
8. Montenegro, M. A., Bülte, M., Trumpf, T. et al.: *J. Clin Microbiol* 6, 1417 (1990).
9. Wells, J. G., Shipman, L. D., Greene, K. D. et al.: *J Clin Microbiol* 5, 985 (1991).
10. Farmer, J. J., Davis, B. R.: *J Clin Microbiol* 4, 620 (1985).
11. March, S. B., Ratnam, S.: *J Clin Microbiol* 5, 869 (1986).
12. Pai, C. H., Gordon, R., Sims, H. V. et al.: *Ann Intern Med* 101, 738 (1984).
13. Wells, J. G., Davis, B. R., Wachsmuth, I. K. et al.: *J Clin Microbiol* 18, 512 (1983).
14. Okrend, A. J., Rose, B. E., Lattuada, C. P.: *J Food Prot* 11, 941 (1990).
15. Szabó, R. A., Todd, E. C., Jean, A.: *J Food Prot* 10, 768 (1986).
16. MacDonald, K. L., O'Leary, M. J., Cohen, M. L. et al.: *JAMA* 259, 3567 (1988).
17. Remis, R. S., MacDonald, K. L., Riley, L. W. et al.: *Ann Intern Med* 101, 624 (1984).

PROGRAMMED CELL DEATH (APOPTOSIS):
ITS VIROLOGICAL AND IMMUNOLOGICAL CONNECTIONS
(A REVIEW)

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(Received October 1, 1991)

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Programmed cell death is a physiological, energy-consuming mechanism leading to suicide of the cell. Cell death is accomplished by the activation of endonucleases that fragment the cell's nuclear DNA. Some tumour cells remain susceptible to programmed death. These are hormone- and growth factor-dependent tumour cells. Hormone or growth factor deprivation induces signals leading to apoptosis. Other tumours gain strong resistance to apoptosis. One of the normal functions of the *bcl-2* gene is to provide longevity to memory B cells. When this gene becomes translocated in follicular B cell lymphomas, it renders lymphoma cells resistant to apoptosis. Latent membrane protein encoded by an EBV gene, either by itself or by amplifying *bcl-2*, enables tumour cells (nasopharyngeal carcinoma; Reed-Sternberg cell of Hodgkin's disease) to resist apoptotic death. Loss of antioncogene *p53* provides for resistance against programmed cell death. Breakdown of resistance to apoptosis in tumour cells can be achieved by oncolytic viruses; generation of lymphotoxin and tumour necrosis factor; monoclonal antibodies; transfection with plasmid vectors carrying *p53*; gamma irradiation; and certain chemotherapeutic agents.

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Cytotoxic lymphocytes

In the late 1960s at the Section of Clinical Tumour Virology and Immunology, Department of Medicine, the University of Texas, M. D. Anderson Hospital, Houston, Texas, we observed lymphocyte-mediated cytotoxicity directed against human tumour cells /1, 2/. Based on morphological observations two types of lymphocyte-mediated cytotoxicity were recognized (Table I). Microphotographs were taken in 1970-1972 (Plate 1).

While these two distinct morphological features of lymphocyte-mediated cytotoxicity were prominent for each of the two lymphocyte subtypes, they were not exclusively practiced by either the large granular lymphocytes (now known as natural killer cells) or by the smaller lymphocytes (now known as T8 cytotoxic cells or immune T cells). Overlap of these features occurred and natural killer cells could kill target cells also by nuclear lysis and vice versa T8 cells also by lysis of cytoplasm. It was during this early observation period that the biological significance of apoptosis was recognized /3-5/. We, however, did not make the connection between certain forms of lymphocyte-mediated target cell destruction and apoptosis until molecular mediators of the phenomena were discovered and were elaborated on elsewhere /6-8/.

It is now known that cytoplasmic lysis is mediated by tubular proteins of lymphocyte origin called perforins /9, 10/. These target cells passively succumb to cytoplasmic leakage. In contrast, the mechanism of nuclear lysis is a complex energy-consuming process in which activated endonucleases of

Table I
Morphological observations of human lymphocyte-mediated cytotoxicity
directed to tumour cells

Target tumour cells	Attacker lymphocytes	Target tumour cell lysis
Allogeneic	Majority: large with light granular cytoplasm	Dissolution of cell membrane and leakage of cytoplasm
	Minority: small with dark cytoplasm	Fragmentation and lysis of nuclear contents
Autologous	Majority: small with dark cytoplasm	Fragmentation and lysis of nuclear contents
	Minority: large with light granular cytoplasm	Dissolution of cell membrane and leakage of cytoplasm

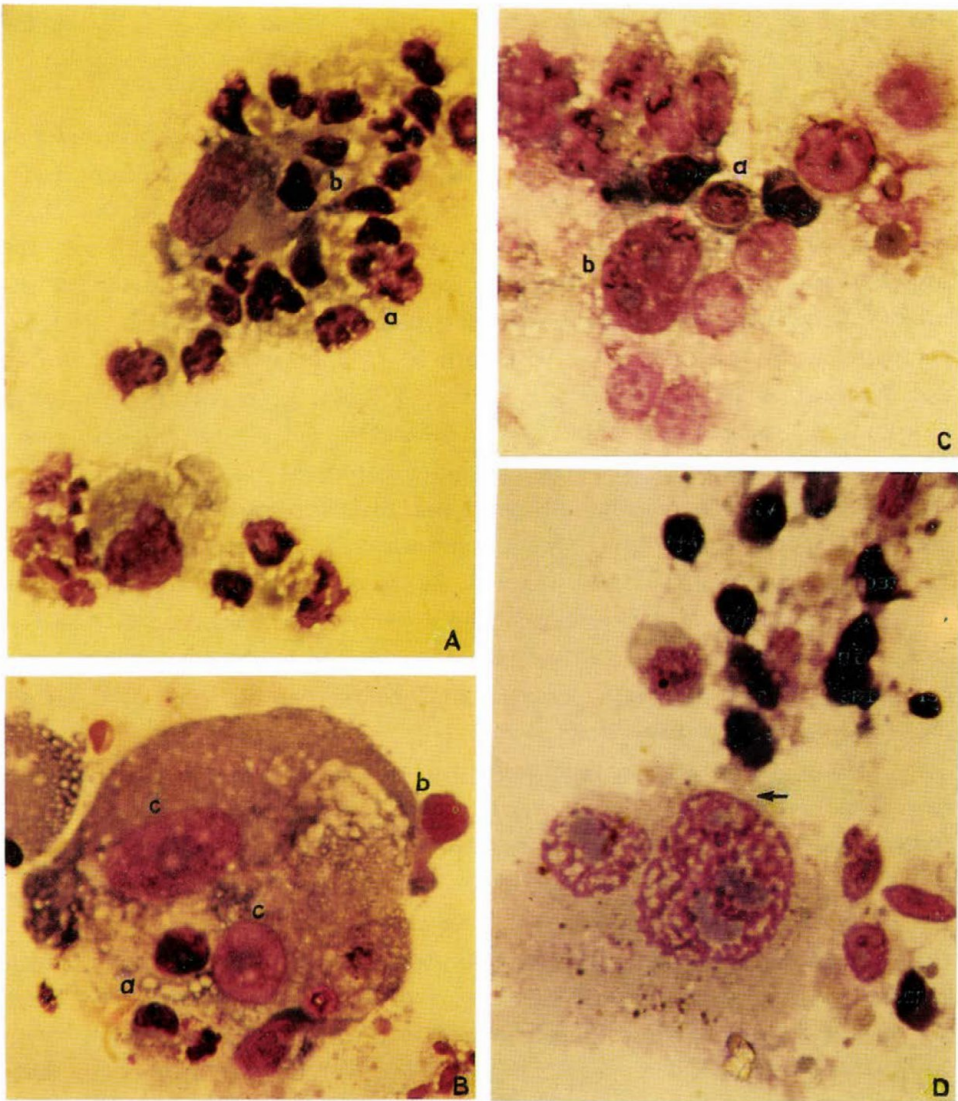


Plate 1. (A) Large lymphocytes lyse the cytoplasm of allogeneic tumour (sarcoma) cell while the nucleus of the target cell remains intact: a — cell debris and denuded nuclei; b — lymphocytes lysing cytoplasm

(B) Allogeneic tumour (sarcoma) cell undergoes cytoplasmic lysis: a — three large lymphocytes destroy cytoplasm; b — as one lymphocyte attaches to cell membrane, major vacuolization of target cell cytoplasm takes place beneath site of attachment; c — target cell nuclei remain intact

(C) After cytoplasmic lysis of numerous target tumour (melanoma) cells: a — three attacker lymphocytes remain tinctorially intact; would commit further acts of cytolysis when in native state were re-exposed to target cells (not shown); b — some denuded target cell nuclei and nucleoli remain intact

(D) After destroying a target tumour cell (melanoma) above, one small autologous lymphocyte makes cytoplasm to cell membrane contact (arrow) with a new target cell which undergoes rapid nuclear lysis

the target cells rapidly condense and fragment nuclear DNA /11, 12/. Cytotoxic T lymphocytes releasing lymphotoxin ($\text{TNF}\beta$) induce fragmentation of nuclear DNA of target cells within minutes of attachment /12/.

Cytolytic granules extracted from the cytoplasm of cytotoxic lymphocytes operating through the release of perforins or purified perforins failed to induce condensation and fragmentation of target cell nuclear DNA /13/. In contrast to osmolytic lysis of target cells effected by perforins, the endonuclease inhibitor Zn^{++} prevented DNA fragmentation, but failed to inhibit ^{51}Cr release from cells killed by perforins /14/. Inhibitors of transcription (actinomycin D; 5,-6,-dichloro-1 β -D-ribofuranosyl benzimidazole) and translation of macromolecular synthesis (emetine; puromycin) prevented cytotoxic T lymphocyte-mediated DNA fragmentation but not cytoplasmic osmotic lysis by perforins /14/. Thus in perforin-induced lysis the target cell is a passive victim, whereas in death due to DNA fragmentation the target cell is an active participant whose programmed self-destruction had been activated by physiologic stimuli represented by certain cytokines or hormones /11, 15/. Lymphotoxin of cytotoxic T cells operates by inducing DNA fragmentation in the nuclei of target cells but these lymphocytes can also release perforins. NK and LAK cells, producers of perforins, also can lyse target cells by nuclear DNA fragmentation /16/.

$\text{TNF}\alpha$ of NK cell or macrophage origin, either secreted or cell membrane associated /17, 18/ is a major inducer of programmed cell death. Hu-r-TNF induces the programmed cell death-associated gene TRPM-2 /19/. Topoisomerase II inhibitors (etoposide; actinomycin D) intensified TRPM-2 mRNA expression, DNA fragmentation and cell death. As $\text{TNF}\alpha$ binds to its receptor it activates second messenger pathways through G proteins, phospholipases and protein kinases and upregulates a multiplicity of cellular genes, among them those of manganese superoxide dismutase, metallothioneine, 2'-5'-oligo-A-synthetase, ICAM-1, IL-6 and 8, c-fos, c-jun, c-myc and those of heat shock proteins. Sustained release of Ca^{++} activates endonucleases resulting in DNA fragmentation /17, 20/. Activation of protein kinase C (for example by IL-1) blocks the activation of endonucleases /15, 17/. Thus while macrophages are a major source of $\text{TNF}\alpha$, they also release IL-1, a potential antagonist of $\text{TNF}\alpha$ -induced apoptosis.

Hormones and growth factors

Corticosteroids like dexamethasone kill lymphocytes by apoptosis /21/. Corticosteroids induce programmed cell death of acute lymphoblastic leukaemia cells so efficiently that acute tumour lysis syndrome may ensue /22/. CLL cells succumb to apoptosis under the effect of corticosteroids /23/. Programmed cell death is physiological in the developing embryo. Fetal hormones and molecular mediators can dissolve a primordial organ anlage for replacement by another permanent organ anlage. For example, the primordial fetal testicle releases the müllerian inhibitory substance, a member of the transforming growth factor- β family which induces self-destruction of the müllerian anlage and thus allows the wolffian anlage to develop /24/. Müllerian inhibitory substance suppresses the growth of ovarian carcinoma cells in culture and may gain therapeutic use against malignant tumours originating from tissues of the müllerian anlage.

Androgen deprivation can lead to programmed death of prostatic cells (including these of prostatic carcinoma?). This process coincides with rapid increase in TGF β expression in the involuting tissues /25/. TGF β is a growth inhibitor of human prostatic carcinoma cells when these cells grow in response to the stimulatory effect of epidermal growth factor. Analogs of luteinizing hormone releasing hormone or somatostatins induce programmed death of hamster pancreatic carcinoma cells /26/. This effect may be due to growth factor deprivation since cholecystokinin or cerulein and secretin stimulate the growth of pancreatic adenocarcinoma cells and somatostatins may act by inhibition of these growth factors /26/. Apoptosis by growth factor deprivation occurs in the IL-3-dependent bone marrow-derived BAF-3 cell line /27/.

Among antagonists of apoptosis are β -endorphins, enkefalin, certain hormones and gastrin-releasing factor (bombesin) /28/. Newcastle disease virus when it activates endorphins /29/, it may antagonize programmed cell death when the virus is used for oncolysis. Bombesin acting as autocrine growth factor for small cell undifferentiated carcinoma cells /30-32/ may also protect these tumour cells from apoptosis. The mycotoxin sporidesmin induces DNA fragmentation resembling apoptosis in lymphocytes and macrophages; the endonuclease inhibitor Zn^{++} protected against sporidesmin-induced apoptosis /33/.

Antibodies and elimination of autoimmune clones

An idiotype antibody directed to membrane IgM of B-CLL cells induces apoptosis. This effect is antagonized by phorbol esters which activate protein kinase C /34, 35/, an enzyme which is often active in protooncogene-oncogene cascade. Antibodies directed to T cell receptor (TCR) also induce apoptosis. This effect is blocked by cyclosporin (similarly the effects of IL-2 are also blocked), whereas dexamethasone-induced programmed cell death is not blocked by cyclosporin but it is antagonized by IL-2 /35/. Clearly more than one pathway for the induction of programmed death of T lymphocytes exist. Monoclonal antibody anti-AP0-1 reacts with a 52kD cell membrane protein of malignant B and T lymphocytes. AP0-1 antigen is overexpressed on T cells transformed by HTLV-1 and these cells succumb to apoptosis upon exposure to this antibody /34/.

Autoreactive T cells are eliminated in the fetal thymus by apoptosis /36/. In the bursa of Fabricius apoptosis is operational during normal and leukaemic B cell development /37/. In the mouse, endogenous retroviral genomic sequences encode "superantigens". Interaction of the T cell receptor domains $\alpha\beta$ with superantigens leads to programmed death of the reactive cells and thus to the elimination of autoreactive T cell clones /38, 39/.

Immortality due to resistance to apoptosis

Cytotoxic T cells make contact with target cells. Human target tumour cells interact with autologous lymphocytes only when the tumour cells express MHC-I and intercellular adhesion molecule-1 (ICAM-1 or CD54) antigens /40, 41/. Lymphocyte function-associated-1 (LFA-1) molecule is the natural ligand for ICAM-1 /42/. Cytotoxic NK cells express ICAM-1 and lyse LFA-1⁺ (and also LFA-1⁻) target cells /43/. Low expression of adhesion molecules characterize naive T cells, whereas memory T cells express increased numbers of LFA-1, CD2 and other adhesion molecules.

EBV-infected and/or immortalized human B cells express adhesion molecules and immune T cells readily destroy them. Malignantly transformed EBV⁺ BL cells escape destruction by immune T cells because of no expression of LFA-1 and 3 and ICAM-1 molecules /44/. Reed-Sternberg (RS) cells of Hodgkin's disease (HD) harbouring episomal genomic EBV sequences of monoclonal origin express latent membrane protein (LMP) /45, 46/. As in EBV⁺ nasopharyngeal carcinoma cells, the expression of LMP prevents differentiation

/47/ and adds to the longevity of LMP⁺ nasopharyngeal carcinoma or RS cells.

The presence of LMP in RS cells is most prominent in the mixed cellularity and nodular sclerosis subtypes of HD (96% and 32%, respectively). It is lowest in the lymphocyte predominant subtype of HD (10%) /45/ which carries the best prognosis. EBNA-2 and EB viral glycoprotein gp350/250 antigens were not expressed in RS cells /46/. EBV-specific DNA sequences occurred in cells of HD lesions in 68% of cases and all LMP⁺ specimens also contained EBV genomic sequences but not all EBV-DNA⁺ HD cells expressed LMP. Among CD30⁺ RS cells of the same patient some were positive, others were negative for LMP /46/.

In a recent essay describing RS cells as natural hybridomas of interdigitating or follicular dendritic cells and B or T cells reactive to retroviral antigens expressed by the former cells, the pathogenic role of EBV was considered to be secondary to a retrovirus in HD /48/. A putative retrovirus was proposed to infect the interdigitating or follicular dendritic cells to form the mononuclear HD cell /48/. The normal predecessors of these cells interact for antigen presentation through intercellular adhesion molecules with lymphocytes /49/. Fusion between these cells was proposed to form RS cells /48/. EBV was envisioned as a secondary pathogen responsible for further gene activation in RS cells after EBV⁺ B cell fused with the retrovirus-infected interdigitating or follicular dendritic cell /48/. While c-myc translocation occurs in EBV⁺ BL cells, in RS cells LMP expression (and bcl-2 activation?) appears to be the prominent event. While LMP may offer a target for cytotoxic lymphocytes /50/, it may also endow the RS cells with longevity.

BL cells with EBV gene expression restricted to EBNA-1 contained no or very low levels of the gene product protein Bcl-2 and succumbed to programmed cell death in media containing 1% fetal calf serum as recognized by acridine orange fluorescence showing nuclear chromatin condensation /51/. BL cells expressing EBNA-1 and 2 and LMP contained large amounts of Bcl-2 detectable by immunoblotting. These cells resisted apoptosis. Transfectants of EBV⁻ to EBV⁺ lymphoma cells showed no bcl-2 induction by EBV genes EBNA-1 to 3 or EBNA-LMP. Transfection with the LMP-1 gene resulted in the production of LMP-1 gene product protein detectable by monoclonal antibodies Cq1-4 and up-regulation of bcl-2. Transfection with the LMP-2 gene did not result in up-regulation of bcl-2 /51/. EBNA 1-3⁺ and LMP-2⁺ clones succumbed to programmed cell death; LMP-1⁺ clones resisted it. Cells transfected with bcl-2

gene did not show LMP-1 expression but expressed Bcl-2 and resisted programmed cell death /51/.

Vector-induced LMP gene of EBV (BNLF-1) induces profound changes in EBV⁻ human lymphoblasts: LFA-1, 3 and ICAM-1 become apparent and F_CRII/CD23 and transferrin receptor appear /52, 53/. The six segments of LMP span the cell membrane and thus may function as ion channels or truncated receptors constitutively activated /52, 53/. Another EBV gene (BHRF-1) shows sequence homology with bcl-2. It is either this gene or the overexpressed endogenous c-bcl-2 that provide for longevity of certain EBV⁺ cells /52/.

The major function of the bcl-2 gene product protein is to protect B cells from succumbing to apoptosis. Human follicular B cell lymphomas show translocation t(14;18)(q32;q21) which involves the bcl-2 gene /54-56/. The gene product protein Bcl-2 is a 24kD mitochondrial integral membrane protein which becomes highly expressed in lymphoid cells carrying t/14;18/; this protein is located at the inner membrane of mitochondria /57/.

The B cell leukaemia lymphoma-2 (bcl-2) gene translocates from 18q21 in 88% of small cleaved cell follicular lymphomas to 14q32. The new location of bcl-2 is the locus of immunoglobulin heavy chain gene at chromosome 14q32 /54-56/. Other genes preferentially translocating to this region are bcl-1 (from 11q13) in small cell lymphocytic lymphomas and in B-CLL; and c-myc (from 8q24) in BL. In some centrocytic lymphomas consisting of cleaved follicular center cells the bcl-1 locus undergoes rearrangement /54, 58/. Occasionally the bcl-2 gene translocates to a light chain locus: t(18;22)(q21;q11) /59/.

The normal gene bcl-2 is most active in pre-B cells. Long-living and recirculating follicular mantle zone cells of IgM/IgD expression express intensely bcl-2 gene protein, whereas centroblasts and centrocytes showing frequent apoptotic death lack bcl-2 gene product protein expression /60/. The translocated gene functions as a fusion gene of bcl-2-Ig. In transgenic mice carrying the fusion gene long-living B cells are generated. B cells carrying bcl-2 first expand polyclonally without cell death. Monoclonal expansion of neoplastic large lymphoma cells takes place thereafter. These cells grow diffusely and show rearranged c-myc /61-63/. EBV-infected human lymphoblasts gain survival advantage upon transfection with bcl-2 genomic sequences /64, 65/.

Monoclonal antibody 6C8 is specific to human bcl-2 gene product protein /66/. Bcl-2 staining revealed high expression in follicular lymphomas; intermediate expression in lymphocytic and mantle zone lymphomas and in CLL.

Variable but low levels were found in T cell lymphomas and plasma cell dyscrasias. Bcl-2 was absent in RS cells of nodular sclerosing HD. Some 30% of HD tissues may show increased bcl-2 expression or even translocation by polymerase chain reaction /67/ but it is not known if RS cells or reactive cells express the gene. CLL cells showed high expression of bcl-2 gene product protein /66/.

When IL-3-dependent pro-B cell line FL5.12 is deprived of IL-3, it dies out. The cells show plasma membrane blebbing, volume loss, nuclear condensation and cleavage of DNA by endonucleases. FL5.12 cells possessing transfected bcl-2 resist apoptosis /68/.

Translocation of bcl-2 in follicular lymphoma is similar to translocation of c-myc in BL. In this latter translocation in BL cells, c-myc moves from 8q24 to 14q32 next to the IgG heavy chain gene acting under the influence of the immunoglobulin enhancer. Normal stimulated T cells express the bcl-2 gene. Introduction of both bcl-2 and c-myc sequences by plasmid vectors into human T cell leukaemia line Jurkat resulted in significantly increased oncogenicity of these cells in athymic mice. Further introduction of a plasmid vector carrying antisense oligonucleotides of the bcl-2 gene abrogated or reduced the newly acquired oncogenicity of transfected Jurkat cells /69-71/.

In human cancers the mutated p53 gene loses its function as "tumour-suppressor gene" /72/. A murine myeloid leukaemic cell line lacks p53. The introduction of p53 into this cell line resulted in prompt programmed cell death. This process was counteracted by IL-6 /73/. Thus loss of p53 may confer resistance to apoptosis and IL-6 may function as a promoter of neoplastic cells by antagonizing programmed cell death. Indeed, AIDS-KS cells /74/ and myeloma cells /75/ utilize IL-6 as autocrine or paracrine growth factor.

Discussion

During the early period when lymphocyte-mediated cytotoxicity directed against tumour cells was observed purely morphologically, the characteristic depiction of programmed cell death was difficult because both perforin-mediated osmotic lysis of cytoplasm and lymphotoxin-mediated lysis of nuclei occurred at the same time. This overlap of cytolytic mechanisms is reflected in Table I. Clear distinction between these two different types of cell lysis could be made only after the discovery and purification of their mole-

cular mediators leading to the recognition that the cell's own activated endonucleases fragment nuclear DNA /76/.

Apoptosis certainly is a major physiologic process in normal development. In the embryo entire primordial organs are eliminated by apoptosis. Encounter of T cell receptor and the $\alpha 3$ domain of class I MHC molecule activates CD8 to deliver a signal to induce programmed death of the reacting T cell, thus eliminating autoimmune clones /77/. One mechanism of oncogenesis is acquisition of resistance to apoptosis. Some neoplastic cells may remain vulnerable to apoptosis; examples are myelogenous leukaemia cells reexpressing antioncogene p53 /73/ or lymphoid cells expressing c-myc /37/. Hormone- or growth factor-dependent tumour cells misconstrue the abrupt withdrawal of the growth promoter as a signal to activate mechanisms of programmed cell death and undergo self-destruction /25, 26/. Thus cytotoxic chemotherapy is not the only mechanism designed for tumour cell killing. Hormonal or growth factor deprivation or antihormones may induce programmed suicide of the cell. Exposure to sublethal doses of gamma radiation may result in DNA repair or in the activation of the mechanisms of apoptosis. Some chemotherapeutic agents kill neoplastic cells through apoptosis /19/. Overexpression of cell surface antigens (vitronectin receptor) in apoptotic cells induces recognition by macrophages that phagocytize and digest cells undergoing apoptosis /78/. Certain haematopoietic colony stimulating growth factors protect against apoptosis /79/.

Anti- $\alpha 3$ monoclonal antibodies protect against apoptosis induced by encounter of CD8 of reactive T lymphocytes and class I MHC of antigen presenting cells /77/. The existence of such antibodies allows autoreactive T cells to prevail.

New therapeutic ideas are introduced by the discovery of monoclonal antibodies that induce apoptosis /34, 35/. Monoclonal antibodies directed to gene product proteins mediating resistance to apoptosis are available for the intracellular detection of these proteins /66/. These antibodies, for example anti-Bcl-2 6C8, should be used in therapeutic trials.

One aspect of viral oncogenesis can be recognized in the activity of certain viral genes whose gene product proteins counteract programmed cell death. LMP induced by EBV in nasopharyngeal and RS cells is currently the best example. EBV viral protein expression regulates sensitivity (only EBNA-1 expressed) and resistance (all 8 EBV latent proteins expressed) of Burkitt's lymphoma cells to apoptosis /80/.

While the normal function of the bcl-2 gene is to preserve the longevity of B (or T?) memory cells, as transgenic mice with amplified Bcl-2 possess immunoglobulin producer B memory cells of extended life span /81/, when this gene is translocated it becomes deregulated and confers resistance to apoptosis to the cells harbouring the translocated gene. Follicular cleaved lymphoma cells expand into huge populations by utilizing this mechanism. Further oncogene activation (for example, that of c-myc) results in monoclonal expansion of highly neoplastic populations of large cell lymphoma /69-71/.

The formation of RS cells can be envisioned as follows /48/. A retrovirally infected mononuclear HD cell is able to replicate. (Note: this retrovirus has not been isolated or identified; its existence is based on indirect evidence /48/.) This cell probably belongs to the family of interdigitating or follicular dendritic cells of lymph nodes /49/. Once this cell fused with a reactive B cell and thus acquired EBV genomic sequences, expression of LMP (and Bcl-2?) renders the cell resistant to apoptosis. This large cell rests and manufactures molecular mediators and immunoglobulins thus evoking an extensive polyclonal cellular response. Among the responding cells prominent are eosinophils mobilized by IL-5 of RS cell origin and B and T lymphocytes. Anti-lymphocyte immunoglobulins released from RS cells deplete the supply of lymphoid cells (perhaps by inducing apoptosis of the reactive cells, a process to which RS cells themselves are resistant). Eventually RS cells survive and the patient dies with lymphocyte-depleted HD.

When HIV envelope protein gp120 is attached to CD4 of helper cells, a signal leading to apoptosis of the target cell is induced (unless syncytium formation occurs allowing for survival of cells and viral replication within). Apoptotic cell death is prevented by anti-gp120 antibody and by tetradecanoyl phorbol-13-acetate which activates protein kinase C /82/. It is entirely feasible to envision oncolytic viruses to exert antitumour effects by inducing, or overriding resistance to, apoptosis (except when virus neutralizing or anti-virus-binding receptor antibodies of the host intervene).

Reinduction of the mechanisms of programmed cell death or overriding the resistance to it in neoplastic cells should empower us with a most efficient antitumour strategy. Judged from its physiologic role in the developing embryo, apoptosis is designed to eliminate not only single cells but large masses of cells as well. Genetically engineered oncolytic viruses used therapeutically /83/; active "tumour-specific" immunotherapy with viral oncolysates resulting in the activation of lymphotoxin producer immune T cells

or TNF α -producer macrophages /84/; deactivation or neutralization by monoclonal antibody of bcl-2 gene product proteins or LMP itself; and re-introduction of lost genetic information deriving from antioncogenes into tumour cells by retroviral plasmid vectors are the new avenues leading to this goal. Chemotherapeutic agents with limited general cytotoxicity but effects focused more on the mechanisms that activate apoptosis in the target tumour cell may be developed /85/.

Acknowledgement. This publication has been submitted in appreciation and acceptance of honorary membership for the author in the Hungarian Society for Microbiology and contains the material intended for presentation at the 1991 annual conference. The author is grateful to Professors Gy. Berencsi and I. Nász for the invitation to publish this material in the *Acta Microbiologica*.

ABBREVIATIONS

BHRF	EBV gene with homology to <u>bcl-2</u>
BL	Burkitt's lymphoma
BNLF	EBV gene encoding LMP
CLL	chronic lymphocytic leukaemia
EBV	Epstein-Barr virus
EBNA	Epstein-Barr nuclear antigen
HD	Hodgkin's disease
HIV	human immunodeficiency virus
HTLV	human T cell leukaemia virus
ICAM	intercellular adhesion molecule
IL	interleukin
LFA	lymphocyte function associated
LMP	latent membrane protein
MHC	major histocompatibility complex
NK	natural killer
RS	Reed-Sternberg
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumour necrosis factor
TRPM	programmed cell death associated gene

REFERENCES

1. Sinkovics, J. G.: *Ann Clin Lab Sci* 16, 488 (1986).
2. Sinkovics, J. G.: In Cory, J. G., Szentiványi, A. (eds): *Cancer Biology and Therapeutics*. Plenum Press, New York, 1987. pp. 225-253.
3. Kerr, J. F. R., Wyllie, A. H., Currie, A. R.: *Br J Cancer* 26, 239 (1972).
4. Wyllie, A. H.: *Arch Toxicol Suppl* 11, 3 (1987).
5. Cotter, T. G., Lennon, S. V., Glynn, J. G., Martin, S. J.: *Anticancer Res* 10, 1153 (1990).
6. Podack, E. R., Olsen, K. J., Lowrey, D. M., Lichtenheld, M.: *Current Topics Microbiology Immunology* 140, 11 (1988).
7. Duke, R. C., Persechini, P. M., Chang, S., Liu, C.-C., Cohen, J. J., Young, J. D.-E.: *J Exp Med* 170, 1451 (1989).

8. Lowrey, D. M., Rupp, F., Aebischer, T., Grey, P., Hengartner, H., Podack, E. R.: *Ann Inst Pasteur Immunol* 138, 296 (1987).
9. Podack, E. R., Dennert, G.: *Nature* 302, 442 (1983).
10. Liu, C.-C., Perussia, B., Cohn, Z. A., Young, J. D.-E.: *J Exp Med* 164, 2061 (1986).
11. Duke, R. C., Chervenak, R., Cohen, J. J.: *Proc Natl Acad Sci USA* 80, 6361 (1983).
12. Paul, N. L., Ruddle, N. H.: *Ann Rev Immunol* 6, 407 (1988).
13. Gronkowski, S. H., Brown, T. C., Masson, D., Tschopp, J.: *J Immunol* 141, 774 (1988).
14. Zychlinsky, A., Zhieng, L. M., Liu, C.-C., Young, J. D.-E.: *J Immunol* 146, 393 (1991).
15. McConkey, D. J., Orrenius, S., Jordal, M.: *Immunology Today* 11, 120 (1990).
16. Leibson, P. J., Windebank, K. P., Vilen, B. J., Regnerus, D. E., Pease, L. R.: *Ann NY Acad Sci* 532, 475 (1988).
17. Larrick, J. W., Wright, S. C.: *FASEB J* 4, 3215 (1990).
18. Perez, C., Albert, I., Defay, K., Zacharides, N., Gooding, L., Kriger, M. A.: *Cell* 63, 251 (1990).
19. Kyprianou, N., Alexander, R. B., Isaacs, J. T.: *J Natl Cancer Inst* 83, 346 (1991).
20. Cotter, T. G., Lennon, S. V., Glynn, J. G., Martin, S. J.: *Anticancer Res* 10, 1153 (1990).
21. Ucker, D. S.: *Nature* 327, 62 (1987).
22. Loosveld, O. J. L., Schouten, H. C., Gaillard, C. A., Blijham, G. H.: *Br J Haematol* 77, 122 (1991).
23. McConkey, D. J., Aguilar-Santelises, M., Hartzell, P., Eriksson, I., Mellstedt, H., Orrenius, S., Jordal, M.: *J Immunol* 146, 1072 (1991).
24. Fuller, Jr. A. F., Krane, I. M., Budzik, G. P., Donahoe, D. K.: *Gynecol Oncol* 22, 135 (1985).
25. Kyprianou, N., Isaacs, J. T.: *Molec Endocrin* 3, 1515 (1989).
26. Szende, B., Zalutsky, A., Schally, A. V.: *Proc Natl Acad Sci USA* 86, 1643 (1989).
27. Rodriguez-Tarduchy, G., Collins, M., Lopez-Rivas, A.: *EMBO J* 9, 2997 (1990).
28. Lynn, W. S., Mathews, D.: *Molecular Cellular Biology Cytokines*. Wiley-Liss, New York, NY 1990. pp. 233-244.
29. Westly, H. J., Kleiss, A. J., Kelley, K. W., Wong, P. K. Y., Yuen, P.-H.: *J Exp Med* 163, 1589 (1986).
30. Cuttitta, F., Carney, D. N., Mulshine, J., Moody, T. W., Fedorko, J., Fischler, A., Minna, J. D.: *Nature* 4, 823 (1985).
31. Carrey, D. N., Cuttitta, F., Moody, T. W., Minna, J. D.: *Cancer Res* 47, 821 (1987).
32. Sethi, T., Rozengurt, E.: *Cancer Res* 51, 3621 (1991).
33. Waring, P., Egan, M., Braithwaite, A., Mullbacher, A., Sjaarda, A.: *In J Immunopharmacol* 12, 445 (1990).
34. Debatin, K.-M., Goldmann, C. K., Banford, R., Waldmann, T. A., Krammer, P. H.: *Lancet* 335, 497 (1990).
35. Zacharchuk, C. M., Mercep, M., Chakraborti, P. K., Simons, S. S., Ashwell, J. D.: *J Immunol* 145, 4037 (1990).
36. Murphy, K. M., Heimberger, A. B., Loh, D. Y.: *Science* 250, 1720 (1990).
37. Neiman, P. E., Thomas, S. J., Loring, G.: *Proc Natl Acad Sci USA* 88, 5857 (1991).
38. Marrack, P., Kuschner, E., Kappler, J.: *Nature* 349, 524 (1991).
39. Antel, J. P., Cashman, N. R.: *Mayo Clinic Proc* 66, 752 (1991).
40. Vanky, F., Wang, P., Patarroyo, M., Klein, E.: *Cancer Immunol Immunother* 31, 19 (1990).
41. Wang, P., Vanky, F., Li, S.-L., Patarroyo, M., Klein, E.: *Cell Immunol* 131, 366 (1990).
42. Dustin, M. L.: *BioEssays* 12, 421 (1990).
43. Yong, K., Khwaja, A.: *Blood Rev* 4, 211 (1991).
44. Gregory, C. D., Murray, R. J., Edwards, C. F., Rickinson, A. B.: *J Exp Med* 167, 1811 (1988).
45. Pallesen, G., Hamilton-Butoit, S. J., Rowe, M., Young, L. S.: *Lancet* 337, 320 (1991).
46. Herbst, H., Dallenbach, F., Hummel, M., Niedobitek, G., Pileri, S., Müller-Lantzsch, N., Stein, H.: *Proc Natl Acad Sci USA* 88, 4766 (1991).
47. Dawson, C. W., Rickinson, A. B., Young, L. S.: *Nature* 344, 777 (1990).
48. Sinkovics, J. G.: *Crit Rev Immunol* 11, 33 (1991).
49. Alavaikko, M. J., Hansmann, M.-L., Nebendahl, C., Parwaresch, M. R., Lennert, K.: *Am J Clin Pathol* 95, 194 (1991).

50. Murray, R. J., Wang, D., Young, L. S., Wang, F., Rowe, M., Kieff, E., Rickinson, A. B.: *J Virol* **62**, 3747 (1988).
51. Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wang, F., Longnecker, R., Kieff, E., Rickinson, A.: *Cell* **65**, 1107 (1991).
52. Williams, G. T.: *Cell* **65**, 1097 (1991).
53. Sugden, B.: *Cell* **57**, 5 (1989).
54. Cotter, F. E.: *Br J Haematol* **75**, 449 (1990).
55. Bakhshi, A., Wright, J. J., Graininger, W., Seto, M., Owens, J., Cossman, J., Jensen, J. P., Goldman, P., Korsmeyer, S. J.: *Proc Natl Acad Sci USA* **84**, 2396 (1987).
56. Raffeld, M., Wright, J. J., Lipford, E., Cossman, J., Longo, D. L., Bakhshi, A., Korsmeyer, S. J.: *Cancer Res* **47**, 2537 (1987).
57. Hockenbery, D., Nunez, G., Millman, C., Schreiber, R. D., Korsmeyer, S. J.: *Nature* **348**, 334 (1990).
58. Williams, M. E., Westermann, C. S., Swerdlow, S. H.: *Blood* **76**, 1387 (1991).
59. Leroux, D., Hillion, J., Monteil, M., Le Marchadour, F., Jacob, M.-C., Sotto, J. J., Larsen, C. J.: *Genes Chromosomes Cancer* **3**, 205 (1991).
60. Korsmeyer, S. J., McDonnell, T. J., Nunez, G., Hockenbery, D., Young, R.: *Current Topics Microbiology Immunology* **166**, 203 (1990).
61. McDonnell, T. J., Nunez, G., Platt, F. M., Hockenbery, D., London, L., McKearn, J. P., Korsmeyer, S. J.: *Molec Cell Biol* **10**, 1901 (1990).
62. McDonnell, T. J., Korsmeyer, S. J.: *Nature* **349**, 254 (1991).
63. Reed, J. C., Cuddy, M., Slabiak, T., Croce, C. M., Nowell, P. C.: *Nature* **336**, 259 (1991).
64. Nunez, G., Seto, M., Seremetis, S., Ferrero, D., Grignani, F., Korsmeyer, S. J., Dalla-Favera, R.: *Proc Natl Acad Sci USA* **86**, 4589 (1989).
65. McDonnell, T. J., Deane, N., Platt, F. M., Nunez, G., Jaeger, U., McKearn, J. P., Korsmeyer, S. J.: *Cell* **57**, 79 (1989).
66. Zutter, M., Hockenbery, D., Silverman, G. A., Korsmeyer, S. J.: *Blood* **78**, 1068 (1991).
67. Stetler-Stevenson, M., Crush-Stanton, S., Cossman, J.: *J Natl Cancer Inst* **82**, 855 (1990).
68. Nunez, G., London, L., Hockenbery, D., Alexander, M., McKearn, J. P., Korsmeyer, S. J.: *J Immunol* **144**, 3602 (1990).
69. Gauwerky, C. E., Huebner, K., Isobe, M., Nowell, P. C., Croce, C. M.: *Proc Natl Acad Sci USA* **86**, 8867 (1989).
70. Vaux, D. L., Cory, S., Adams, J. M.: *Nature* **335**, 440 (1988).
71. Reed, J. C., Cuddy, M., Halder, S., Croce, C., Nowell, P., Makover, D., Bradley, K.: *Proc Natl Acad Sci USA* **87**, 3660 (1990).
72. Hollstein, M., Sidransky, D., Vogelstein, B., Harris, C. C.: *Science* **253**, 49 (1991).
73. Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., Oren, M.: *Nature* **352**, 345 (1991).
74. Miles, S. A., Rezai, A. R., Salazar-Gonzales, J. F., Vander, M. M., Stevens, R. H., Logan, D. M., Mitsuyasu, R. T., Taga, T., Hirano, T., Kishimoto, T. et al.: *Proc Natl Acad Sci USA* **87**, 4068 (1990).
75. Levy, Y., Tsapis, A., Brouet, J.-C.: *J Clin Invest* **88**, 696 (1991).
76. Arends, M. J., Morris, R. G., Wyllie, A. H.: *Am J Pathol* **136**, 593 (1990).
77. Sambhara, S. R., Miller, R. G.: *Science* **252**, 1424 (1991).
78. Savill, J., Dransfield, I., Hogg, N., Haslett, C.: *Nature* **343**, 170 (1990).
79. Williams, G. T., Smith, C. A., Spooncer, E., Dexter, T. M., Taylor, D. R.: *Nature* **343**, 76 (1990).
80. Gregory, C. D., Dive, C., Henderson, S., Smith, C. A., Williams, G. T., Rickinson, A. B.: *Nature* **349**, 612 (1991).
81. Nunez, G., Hockenbery, D., McDonnell, T. J., Sorensen, C. M., Korsmeyer, S. J.: *Nature* **353**, 71 (1991).
82. Terai, C., Kornbluth, R. S., Pauza, C. D., Richman, D. D., Carson, D. A.: *J Clin Invest* **87**, 1710 (1991).
83. Martuza, R. L., Malick, A., Markert, J. M., Ruffner, K. L., Coln, D. M.: *Science* **252**, 854 (1991).
84. Sinkovics, J. G.: *Internatl Rev Immunol* **7**, 259 (1991).
85. Fizoki, H., Shimada, H., Osaka, F., Sakurada, T.: *J Immunol* **141**, 1652 (1988).

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